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ENVIRONMENTAL MICROBIOLOGY

Human-Associated Methicillin-Resistant *Staphylococcus aureus* from a Subtropical Recreational Marine Beach

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Abstract Reports of *Staphylococcus aureus* including methicillin-resistant *S. aureus* (MRSA) detected in marine environments have occurred since the early 1990s. This investigation sought to isolate and characterize *S. aureus* from marine waters and sand at a subtropical recreational beach, with and without bathers present, in order to investigate possible sources and to identify the risks to bathers of exposure to these organisms. During 40 days over 17 months, 1,001 water and 36 intertidal sand samples were collected by either bathers or investigators at a subtropical recreational beach. Methicillin-sensitive *S. aureus* (MSSA) and MRSA were isolated and identified using selective growth media and an organism-specific molecular marker. Antimicrobial susceptibility, staphylococcal cassette chromosome *mec* (SCC*mec*) type, pulsed-field gel electrophoresis (PFGE) pattern, multi-locus sequence type (MLST), and

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staphylococcal protein A (*spa*) type were characterized for all MRSA. *S. aureus* was isolated from 248 (37 %) bather nearby water samples at a concentration range of <2–780 colony forming units per ml, 102 (31 %) ambient water samples at a concentration range of <2–260 colony forming units per ml, and 9 (25 %) sand samples. Within the sand environment, *S. aureus* was isolated more often from above the intertidal zone than from intermittently wet or inundated sand. A total of 1334 MSSA were isolated from 37 sampling days and 22 MRSA were isolated from ten sampling days. Seventeen of the 22 MRSA were identified by PFGE as the community-associated MRSA USA300. MRSA isolates were all SCC*mec* type IVa, encompassed five *spa* types (t008, t064, t622, t688, and t723), two MLST types (ST8 and ST5), and 21 of 22 isolates carried the genes for Panton–Valentine leukocidin. There was a correlation (r=0.45; p=0.05) between the daily average number of bathers and *S. aureus* in the water; however, no association between exposure to *S. aureus* in these waters and reported illness was found. This report supports the concept that humans are a potential direct source for *S. aureus* in marine waters.

Introduction

Staphylococcus aureus is an opportunistic pathogen that can be found as a commensally colonizing organism in approximately 30 % of humans [1]. While some individuals can be long-term carriers of *S. aureus*, colonizations are often transient in nature [2]. *S. aureus* can typically be found in the anterior nares of colonized individuals [3, 4]. However, organisms may also be found in other areas such as the skin, axilla, perineum, and pharynx [3, 5]. Infections that are caused by *S. aureus*, and specifically methicillin-resistant *S. aureus* (MRSA), are primarily of the skin and soft tissues. However, these bacteria are also capable of causing serious systemic infections, including sepsis, pneumonia, endocarditis, and osteomyelitis [6, 7], and these infections are usually caused by a colonized individual's own organisms [8, 9]. Historically, MRSA infections were primarily associated with hospital or health care settings but, with the emergence of potentially more pathogenic community-associated organisms (CA-MRSA), in the past 15 to 20 years, infections are no longer limited to health care settings and now are often seen as community-associated infections in healthy individuals, both young and old [10–13].

Infections with CA-MRSA have been reported after normal activities and contacts of healthy individuals in high-density environments. These include infections acquired after use of common areas such as locker rooms or athletic facilities or in other environments where people are in close contact and encounter shared surfaces [14–17]. Spread is facilitated by the ability of *S. aureus* to survive on surfaces for weeks to months [18], and studies have shown a seasonal increase in MRSA infections during warmer months [19–21]. The CA-MRSA strain USA300 is particularly adept at surviving and spreading on surfaces in a discrete setting or population [18, 22, 23] and MRSA has been found in a variety of non-clinical environments utilized by people [14, 24–30]. The increased incidence of infections caused by CA-MRSA/USA300 has led to inquiries regarding environmental factors associated with human exposure and infection [14, 31, 32].

Contacts at recreational marine beaches and waters have long been implicated as a possible source for *S. aureus* skin infections. In a retrospective epidemiological study conducted in Hawaii that included microbiological monitoring of coastal waters, contact with marine water was associated with *S. aureus* skin infections in a pediatric population (ages 4 months to 16 years), and both methicillin-sensitive *S. aureus* (MSSA) and MRSA were isolated during this study [33, 34]. In the past several years, MSSA and MRSA have been isolated from marine waters and sand from multiple locations including Hawaii [28, 33–35], Egypt [36], Pacific Northwest [26, 27], California [25, 37], and South Florida [24, 38], as well as fresh water sources from the Northwestern USA [26] and Hawaii [35]. With the exception of the studies done at the Northwest US coast, these studies have primarily described *S. aureus* in the context of methods of isolation, associated environmental conditions, or temporal and spatial relations and did not attempt to characterize these organisms. The MRSA isolated from the Northwest US coast were related to hospital-associated strains [27] or organisms primarily associated with animal sources [26]. Recent studies characterizing both colonizing and shed MSSA and MRSA have shown that both adults and toddlers in diapers shed their own colonizing organisms into marine waters in pool settings [39–41] supporting that people could be a source of *S. aureus* in this environment. In addition, *S. aureus* can survive in seawater for multiple days [42] and is capable of replicating in sand [43]. Therefore, a recreational beach setting could provide a transmission pathway for exposure and possible infection with *S. aureus*.

To further explore marine environments as a potential transmission pathway for MSSA and MRSA infection and to determine risk of infection to bathers after exposure to *S. aureus* in marine waters, this investigation examined the distribution and potential sources of MSSA and MRSA in a subtropical recreational beach environment. Additionally, MRSA isolates were characterized by genotypic analyses and antimicrobial profiling to ascertain their potential virulence and spread.

Materials and Methods

Sample Types and Strategy

Two different water sample types and beach sands were collected from a popular, well-characterized recreational

South Florida marine beach lacking a point source for sewage contamination during December 2007 through August 2009 (Electronic Supplementary Material (ESM) 1, 2a, b; images of study beach). The water collected included bather nearby water (BNW) samples (described below) obtained by study participants to assess the risk of *S. aureus* exposure to a bather using marine waters. Ambient water samples and sand were collected by a study team to determine the conditions in which MSSA and MRSA might be routinely found at this location.

Bather Nearby Water Samples

A total of 668 BNW samples were collected by bathers in conjunction with the Beach Environmental Assessment and Characterization Human Exposure Study (BEACHES) on 15 study days from December 2007 through June 2008. This study was approved by the Florida Department of Health Internal Review Board (IRBH07164) and the University of Miami Internal Review Board (IRB 20070306) and was conducted to assess the risk to a bather associated with exposure to potentially pathogenic bacteria from the water immediately surrounding them and included extensive environmental sampling for indicator organisms and other pathogens [44]. Briefly, 1,303 adult regular bathers were randomly assigned to bather or non-bather groups. Bathers went into the water whereas non-bathers stayed on the beach. All participants answered three questionnaires (detailed below) that allowed for self-report of illness before and after exposure. Study participants randomized to the bather group spent 15 min in the water and submerged themselves three times. A BNW sample was collected at knee-deep water (approximately 0.5 m) using a 5-L sterilized plastic container either after the first, second, or third submersion depending upon random lane assignments. Sampling points ranged from 10 to 40 m offshore perpendicular to the shoreline depending on tidal stage. Approximately 200 mL of the BNW samples were analyzed for the presence of MSSA and MRSA as outlined below.

The three questionnaires included: (1) a baseline health questionnaire administered between 1 and 7 days prior to the beach study day; (2) an exposure day questionnaire to capture any changes in health status and other potential covariates, since the initial interview and prior to randomization; and (3) a follow-up questionnaire administered 7 days after exposure to assess the development of any illness and the potential covariates that may have occurred since the exposure day. All questionnaires were administered by study team members in person or by telephone. The disease endpoints of interest were self-reported symptoms consistent with: gastrointestinal illness, acute febrile respiratory illness (i.e., ICD9 461–466, 480), eye or ear infections, and skin infections (For further explanation of methods, see [44]).

Ambient Water Samples

A total of 333 water samples were collected by investigators at the study beach at predetermined times, locations, and depths as described below over a 3-month period from August through October 2008. Sample days were Thursdays, Saturdays, and a single Monday holiday, chosen as predicted representatives of lower user density days (Thursdays) and higher user density days (Saturdays and holiday). Samples were collected three times per day at 9:00–10:00 am, 11:30–12:30 pm, and 3:00–4:00 pm. Due to inclement weather on two study days, nine planned samples were not collected. All samples were collected by study team members who were negative for *S. aureus* colonization or infection.

The study beach, an area of approximately 30,000 m^2, was divided into three segments (ESM 1; satellite image of study beach) in order to collect samples representative of local conditions. Sampling was performed along established transect lines (T1, T2, T3) running perpendicular to the shoreline and ranging from 5 to 50 m offshore to reach the desired water depths for collection: 0.3 m (shin-deep) and 1 m (waist-deep). The distance from the shore to the established water depth for collection varied depending upon tidal conditions (ESM 2c, d: photographs of study beach). Upon reaching the assigned transect point, the sampler briefly submerged their arms, waited 1 min for sediment to settle, opened a pre-sterilized 2.3 L polypropylene bottle upcurrent and away from their body, rinsed three times at an elbow depth (0.3 m) while facing into the current, and then filled the bottle. Data recorded for each sampling event included: the Global Positioning System coordinates of the sampling points along the established transects, tidal and weather conditions, the time interval between the sampling, and any recent storm event within the previous 24 h. Visual observances (including activities on the beach or in the water, or noticeable discharges into the water) were also documented. Bather density was assessed using visual counts of persons in the water or on the beach within the study area at the time when the water samples were collected.

Sand Samples

Thirty-six sand samples were collected over 6 days between July and August 2009 from three transects: dry (just above the high tide line), intermittently wet (in the swash zone), and inundated (continuously underwater). All sand samples were collected along a defined intertidal zone. Two samples were collected per transect per sampling day within 1 h after high tide. Sand was collected with sterile instruments and processed as described previously [45].

Water Sample Processing

All water samples were collected into 2.3 or 5 L sterile bottles filled at elbow depth (0.3 m) and sealed onsite. Bottles were transported to the laboratory in coolers containing cold packs or maintained at 4 °C and processed within 6 h of collection. *S. aureus* was enumerated from water using 50 and 5 mL filtration volumes and from sand using an elution method (100 mL extraction volume followed by 0.5 or 5 mL filtered for dry sand and 5 and 50 mL filtered for other sands). Filtrates were passed through 0.45 μm sterile Metricel® filters (Pall Corp., Ann Arbor, MI) using a standard membrane filter method [46] as described [40] and plated on selective media for isolation and subsequent genetic characterization of MSSA and MRSA populations as outlined below. Control field blanks consisting of sterile water samples transferred into sample bottles and treated as experimental samples were processed once daily at the sample collection sites and three times per day in the laboratory to confirm that there was no contamination from personal flora occurring.

Bacterial Isolation and Identification

S. aureus from all water sample collections were isolated and identified as described [41]. Samples were collected onto filters using a standard membrane filtration procedure [46]. Briefly, aliquots of water were filtered through 0.45 μm sterile Metricel® filters to capture bacteria. Filters were placed on Baird Parker agar with egg yolk tellurite enrichment (Becton, Dickinson and Company, Sparks, MD) and incubated aerobically at 37 °C for 24 to 48 h. After incubation, colonies found to be black, shiny, convex, 2–5 mm in diameter, and surrounded by clear zones were considered presumptive *S. aureus* and subjected to further confirmatory testing. Presumptive positive isolates were transferred to mannitol salt agar (BD) for determination of mannitol fermentation and incubated aerobically at 37 °C for 16–24 h. Mannitol-fermenting isolates were transferred to trypticase soy agar with 5 % sheep blood (TSA II™; BD) and subjected to latex agglutination testing for clumping factor and protein A using the BACTiSTAPH® Latex Agglutination Test (Remel via Thermo Fisher Scientific, Lenexa, KS). Whole cell extract of each positive isolate was obtained using the Amplicor MTB Sputum Specimen Preparation Kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's recommendations and served as the template DNA for quantitative real-time PCR (qPCR) confirmation of the *S. aureus*-specific *gyrA* gene as previously described [47], with the minor modification that 1 μL of crude lysate was used as template for qPCR reactions instead of purified chromosomal DNA.

S. aureus and MRSA Characterization: PCR Determination for Select Genes

Bacterial isolates determined to be *S. aureus* were subjected to additional qPCR to test for the methicillin resistance gene, *mecA*, as a marker for MRSA as described [47]. All MRSA and 531 MSSA were subjected to additional qPCR to test for the presence of genes encoding factors known to be associated with virulent *S. aureus*: exfoliative toxins A and B (*eta*, *etb*); staphylococcal enterotoxins A, B, G, K, M, and Q (*sea*, *seb*, *seg*, *sek*, *sem*, *seq*); toxic shock syndrome toxin-1 (*tst*-1); Panton–Valentine leukocidin (PVL; *lukS-PV* and *lukF-PV*); leukocidin (*luk*DE); alpha, beta, and gamma hemolysins (*hla*, *hlb*, and *hlg*ABC); chemotaxis inhibitory protein; and arginine catabolic mobile element arginine deiminase (*arcA*). The MSSA tested included 120 MSSA collected from the ambient water samples and 399 MSSA collected from the bather nearby water samples. The MSSA were selected as a representative sample consisting of one to three organisms isolated from each MSSA positive sample and chosen to include a representative of each hemolytic phenotype based upon growth on trypticase soy agar with 5 % sheep blood. Genomic DNA was extracted from MRSA and select MSSA isolates using the DNeasy® Blood and Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's protocol, with lysis buffer (20 mM Tris–Cl, pH 8, 2 mM EDTA, 1.2 % Triton X-100) containing 0.25 mg/mL lysostaphin (Ambi Products LLC, Lawrence, NY). Purified DNA was evaluated and quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and 5 ng was used as template in 25 μL qPCR reactions as previously described [47]. Beacon Designer™ (Premier Biosoft, Palo Alto, CA) was used to design oligonucleotide primers for this study. Optimized annealing temperatures and control strains used for qPCR as well as references for previously designed primers are listed in ESM 3 (qPCR primers). The identification of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) for all isolates was determined via PCR using methods and primers by Tristan et al. [48].

SCC*mec* Typing, *spa* Typing, MLST, and PFGE of MRSA Isolates

SCC*mec* typing was done as described [49, 50] and the Network on Antimicrobial Microbial Resistance in *S. aureus* repository strain NRS386 was used as a SCC*mec* type IVa control. *spa* typing was performed for all MRSA as described [51]. *spa* sequences were analyzed and assigned *spa* types using the Egenomics *spa* type tool (http://tools.egenomics.com) and using a numerical system (e.g., *spa* type 1 [52]). Ridom database equivalents were identified using the Ridom *Spa*server website developed by Ridom GmbH and curated by SeqNet.org

(http://spa.ridom.de) [53] and were reported using a numerical system preceded by a "t" (e.g., t008).

MLST was determined for all MRSA obtained for this study using previously described oligonucleotide primers and methods for seven housekeeping genes: carbamate kinase (*arc*), shikimate dehydrogenase (*aro*), glycerol kinase (*glp*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*), and acetyl coenzyme A acetyltransferase (*ygi*) [54]. DNA sequence was generated by Eurofins MWG Operon, (Huntsville, AL) and analyzed using an online database (http://saureus.mlst.net).

All MRSA isolated for this study and a control population of 32 human clinical MRSA obtained for a separate study were analyzed by pulsed-field gel electrophoresis. The clinical controls were isolated from infected skin lesions or serious systemic infections from 32 separate patients from local hospitals or clinics and were selected based on matching *spa* types with the study MRSA. PFGE was performed as previously described [55]. Briefly, cells from a 5-mL overnight culture were pelleted, embedded in agarose plugs, and lysed. Plugs were digested overnight with 30 U of *Sma*I (Roche, Indianapolis, IN), and digested DNA separated on a 1 % SeaKem agarose gel using a CHEF-DRII pulsed-field electrophoresis system (Bio-Rad). Electrophoresis was carried out at 6 V for 21 h with a ramped pulse time of 5 to 40 s in 0.5× Tris–borate–EDTA buffer (14 °C). DNA standards were prepared from *Salmonella enterica* serotype Braenderup H9812 as previously described [56]. Cluster analysis was performed with BioNumerics software (Applied Maths Scientific Software Development, Sint-Martens-Latem, Belgium) using Dice coefficient and the unweighted pair group method. Optimization settings for dendrograms were 3 % with a band tolerance of 3 %.

Antimicrobial Susceptibility Tests

The Sensititre® MIC and Breakpoint Susceptibility panel (TREK Diagnostic Systems, Inc., Cleveland OH) was used for the susceptibility testing of all MRSA isolates. Briefly, three to five colonies from an agar plate were added to sterile water and adjusted to a 0.5 McFarland Standard. A 30 μL inoculum was transferred to 10 mL Mueller–Hinton broth with TES buffer per manufacturer's guidelines. The plates were inoculated with 50 μL of the broth suspension using the Sensititre AutoInoculator® (TREK). The plate wells were covered with an adhesive seal and then incubated at 35 °C for 24 h. The minimal inhibitory concentration was recorded as the lowest concentration of antimicrobial that inhibited visible growth. Guidelines supplied by the Clinical and Laboratory Standards Institute were used including all recommended controls, to determine susceptibility and resistance. Antibiotics used in susceptibility tests included penicillin, ampicillin, oxacillin, erythromycin, clindamycin, vancomycin, trimethoprim/sulfamethoxazole, ciprofloxacin, levofloxacin, moxifloxacin, tetracycline, synercid, rifampin, linezolid, daptomycin, gentamicin, and chloramphenicol.

Statistical Analysis

Microsoft Office Excel 2007 and SigmaPlot 11 were used for data management and statistical analyses for microbial concentrations. Differences of the mean microbial concentrations were evaluated using *t* tests for normally distributed data. The Mann–Whitney rank sum test was used in lieu of the *t* test in the cases where normality tests failed. Pearson Product Moment Correlations for normal distributed data and Spearman Rank Order Correlations for non-normal distributed data were used to determine associations between number of bathers and microbial concentration. The analysis of the BNW samples included a comparison with epidemiologic data, therefore also required the use of SAS and StatXact statistical packages as previously described [44, 57]. The bivariate logistic regression was used to evaluate a dose–response relationship between microbial concentrations and bathers' self-reported skin illness.

Results

MSSA and MRSA Isolated from the Bather Nearby Water

Bather nearby water samples were collected by study participants as part of larger study to evaluate the risk to a bather associated with exposure to potentially pathogenic bacteria from the water immediately surrounding them. Of the1,303 participants in the BEACHES study [44], 668 were randomized to go into the water and collect samples for isolation of bacteria. *S. aureus* was isolated from 248 of the 668 BNW samples (37 % [57]) at a range of 2–780 CFU/100 mL water (Table 1). MSSA were isolated on all 15 collection days. MRSA was isolated on 6 of 15 days, from seven of the water samples (3 %) at a range of 2–68 CFU/100 mL water. A total of 1,050 MSSA and 17 MRSA isolates were collected and analyzed from BNW samples. A minimum of one and a maximum of 20 bacterial isolates were collected from any BNW sample containing MSSA or MRSA. Of the positive samples, 3.7 % produced ten or greater isolates each and less than 1 % produced 20 (n=3). A single sample contained both MSSA (n=9) and MRSA (n=1). Ninety-seven percent of the presumptive positive *S. aureus* that were subjected to confirmatory qPCR were confirmed MSSA or MRSA.

No association was identified between exposure to *S. aureus* in the water and any self-report of illness 7–10 days after participation among all individuals whose water samples contained bacteria. When the analysis was restricted to only those samples that were positive for *S. aureus*, there

Table 1 Summary of characteristics of all water and sand samples obtained

Samples	Bather nearby water	Ambient water	Sand
Collected by	Bathers[a]	Investigators	Investigators
Monitoring period	15 days from Dec. 2007 to June 2008	19 days from July 2008 to Sept. 2008	6 days from Aug. 2009
Sampling time	7:44 am to 11:52 am	9:00 am to 10:00 am 11:30 am to 12:30 pm 3:00 pm to 4:00 pm	am
Water depth	0.5 m (knee depth)	0.3 and 1 m (shin and waist depth)	NA
Sampling site	Single site per individual	Along three transect lines	Intertidal zone
Total number of samples	668	333	36
Number of days with *S. aureus* isolated	15	18	5
Number/% *S. aureus*-positive samples (MSSA or MRSA)	248/37 %	102/31 %	9/25 %
Total MSSA isolates collected	1,050	272	12
Number of samples with MSSA	242	101	8
Number of days with MSSA	15	18	4
Concentration range	<2 to 780 CFU/100 mL	<2 to 260 CFU/100 mL	ND
Total MRSA isolates collected	17	2	3
Number of samples with MRSA	7	2	3
Number of days with MRSA	6	2	2
Concentration range	<2 to 68 CFU/100 mL	<2 to 20 CFU/100 mL	ND

[a] Individual bathers who participated in environmental epidemiological studies [46]

NA not applicable, *ND* not done

was also no significant association between increasing counts of bacteria and any of the reported illnesses.

MSSA and MRSA Isolated from Ambient Water

Ambient water samples were collected to evaluate the presence and characteristics of *S. aureus* isolated from these marine waters under normal use conditions. Of the 333 ambient water study samples collected on 19 study days by investigators, 102 (31 %) were determined to contain *S. aureus* at a range of 2–260 CFU/100 mL water, and two (approximately 1 %) had MRSA at a range of 2–20 CFU/100 mL water. MSSA and/or MRSA were isolated each day except one. A total of 272 MSSA and 2 MRSA were isolated from these samples. The percentage of water samples that contained *S. aureus* was not significantly different between the three collection transects: T1, 30.4 % (n=34); T2, 39.2 % (n=40); T3, 30.4 % (n=31), (p=0.33); between shin-deep water (0.3 m) at 47 % (n=48) and waist-deep water (1 m) at 53 % (n=53), (p=0.71); or between the three sampling times: 9:00 am, 33.3 % (n=34); 11:30 am, 36.3 % (n=37); 3:00 pm, 30.3 % (n=31), (p=0.83). The range of positive samples per collection day, shown in Fig. 1, was from 0 % (on a Thursday; September 11, with 18 samples collected in inclement weather and few beachgoers) to

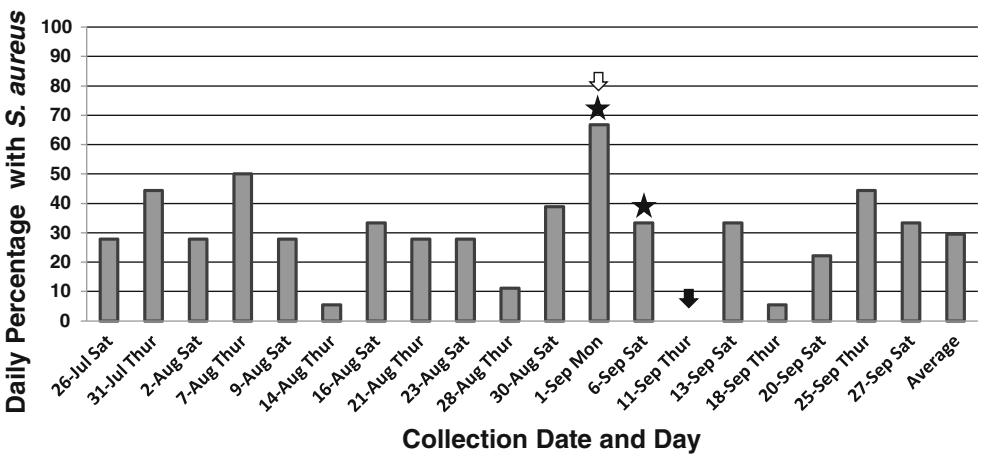

Fig. 1 Daily percentage of ambient water samples from which MSSA or MRSA were isolated. *Stars* denote the days that MRSA were isolated. *Open arrow* denotes holiday and *solid arrow* indicates day with inclement weather

67 % (on a holiday; September 1, with a crowded beach), with an average of approximately 31 % positive samples across all days. A minimum of one and a maximum of ten bacterial isolates were collected from any ambient water sample containing MSSA or MRSA. Of the positive samples, 10.5 % produced six or greater isolates each and 4 % produced ten. A single sample contained both MSSA (n=2) and MRSA (n=1). Ninety-seven percent of the presumptive positive *S. aureus* that were subjected to confirmatory qPCR were confirmed MSSA or MRSA.

In order to evaluate the possible relationship between bather density and the presence of *S. aureus* in the water, the *S. aureus* counts at the specific collection times for the ambient water study were compared with bather densities for that same time. There were from 0 to 131 bathers observed during manual counts by the investigation team at any given time during the samplings. The instantaneous number of bathers did not strongly correlate with the instantaneous concentrations of *S. aureus* isolated from the water (r=0.12; p=0.03) or the daily percentage of *S. aureus*-positive samples (r=0.32; p=0.18; data not shown). The daily geometric mean of *S. aureus* and the average number of people in the water (1 to 44 bathers) during each of the sampling days correlated (r=0.45; p=0.05; Fig. 2). Water quality data, and human usage counts, can be highly variable throughout the course of a day. The averaging provided by computing the geometric mean and average bather counts permits for the suppression of some of this variability, to allow for the observation of trends.

MSSA and MRSA Isolated from Beach Sand

Intertidal zone sand samples were collected to evaluate the presence and characteristics of *S. aureus* isolated from this location under normal use conditions. From the beach sand study samples, a total of eight sand samples were positive for MSSA and three were positive for MRSA (Table 1). Organisms were isolated on four of the six sampling days from dry and intermittently wet sand zones. No *S. aureus* was found in inundated sand. Six of the eight MSSA and all of the three MRSA samples were found in the dry sand. Two MSSA samples were obtained from intermittently wet sand. A minimum of one and a maximum of four bacterial isolates were collected from any sand sample containing MSSA or MRSA. A single sample contained both MSSA (n=1) and MRSA (n=1). Of the presumptive positive *S. aureus* that were subjected to confirmatory qPCR, 48 % were confirmed MSSA or MRSA.

Antimicrobial Susceptibilities and Genetic Characterization of *S. aureus* and MRSA

In total, 1,356 *S. aureus* isolates were collected for these studies from a total of 340 water and 9 sand samples. A total of 1,050 MSSA and 17 MRSA were isolated from BNW samples, 272 MSSA and 2 MRSA from ambient water samples, and 12 MSSA and 3 MRSA from beach sand samples. In total, 22 MRSA isolates were collected from 12 samples collected on 10 out of the combined 40 sampling days. Specifically, 17 MRSA came from seven BNW

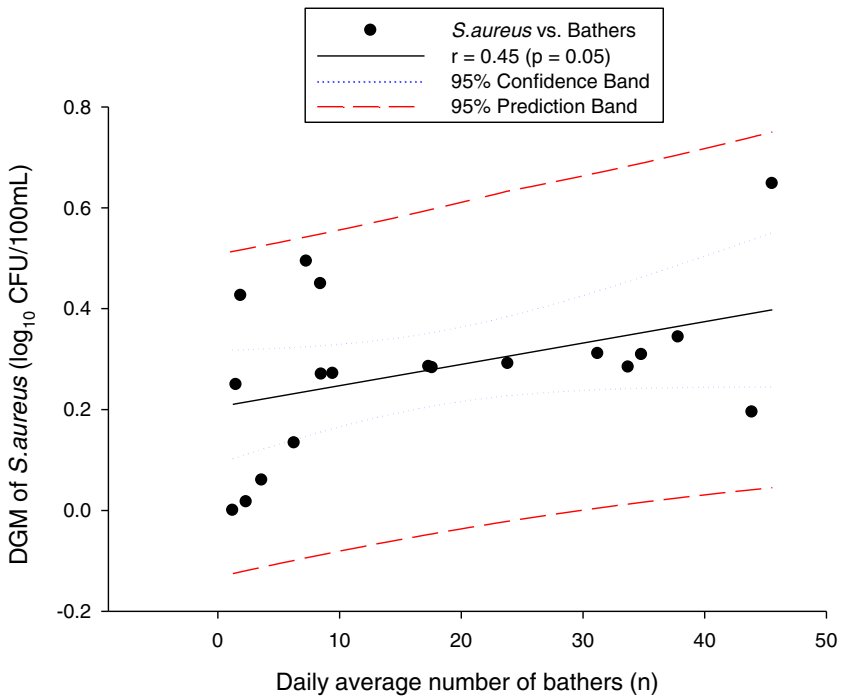

Fig. 2 Daily geometric mean (DGM) of *S. aureus* in recreational marine waters correlated with the average number of bathers in ambient water. The presence of *S. aureus* in the water correlated with the average number of people in the water by direct visual observation

samples collected on 6 (of 15) days, 2 MRSA came from two ambient water samples from 2 (of 19) days, and 3 MRSA came from three sand samples collected on 2 (of 6) days (Table 1, Fig. 3). All MRSA were subjected to analysis including antimicrobial susceptibilities, SCC*mec* typing, *spa* typing, MLST determination, PFGE, and toxin gene profiles to access their pathogenic potential and possible sources. Results of the analyses for all MRSA are shown in Fig. 3. All MRSA were determined to be SCC*mec* type IVa and encompassed five *spa* types (t008, t064, t622, t688, t723) and two MLST types (ST8 and ST5). PFGE results identified 17 of 22 MRSA isolated from seven separate samples collected on 6 days as CA-MRSA strain USA300; three MRSA collected from two samples on separate days were greater than 90 % similar to USA700; and one MRSA isolate was indistinguishable from USA100. When the PFGE results for study isolates were compared to results from a population of clinical MRSA isolated locally from infected skin lesions or obtained from serious systemic infections, 18 (82 %) were identical to clinical isolates, three isolates showed greater than 95 % similarity, and the remaining MRSA was 85 % similar to a clinical isolate (Fig. 4). Toxin gene profiles revealed that 21 (95 %) study MRSA were positive for the gene for PVL, a known virulence factor and marker for potentially virulent organisms [58, 59]. No MRSA were positive for *tst*-1, *eta*, *etb*, *sea*, or *seb*. Two MSCRAMM patterns were determined for all MRSA. BLP115 carried genes *eno*, *ebp*S, *fib*, *clf*A, and *clf*B.

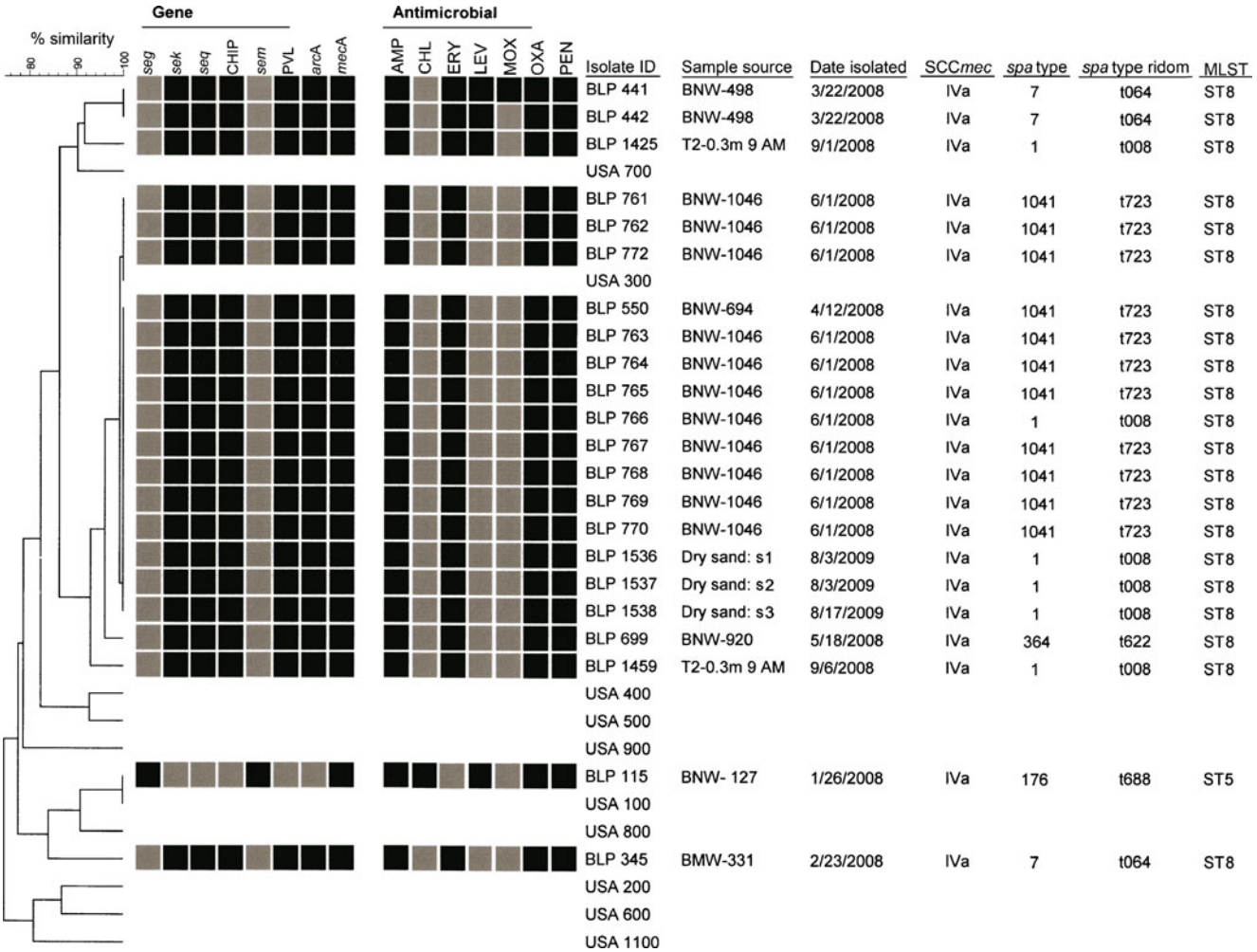

Fig. 3 PFGE-based dendrogram, genetic profiles, antimicrobial sensitivities, and sample sources of MRSA study isolates. Bionumerics software was used to generate a dendrogram based on PFGE results as compared to USA prototype controls. Sample sources: *BNW*, bather nearby water; *T*, transect/ambient water. *Black boxes* indicate an isolate was positive for a gene or resistant to an antimicrobial. *Gray boxes* represent negative genetic results or antimicrobial sensitivity. Genes are designated as follows: *seg*, *sek*, *sem*, and *seq*, staphylococcal enterotoxins G, K, M, and Q; *PVL*, Panton–Valentine leukocidin; *CHIP*, chemotaxis inhibitory protein; and *arcA*, arginine catabolic mobile element arginine deiminase. Antimicrobials are designated as follows: *AMP*, ampicillin; *CHL*, chloramphenicol; *ERY*, erythromycin; *LEV*, levofloxacin; *MOX*, moxifloxacin; *OXA*, oxacillin; and *PEN*, penicillin. All isolates were negative for exfoliative toxins A and B, (*eta*, *etb*), staphylococcal enterotoxins A and B (*sea*, *seb*), and toxic shock syndrome toxin (*tst*-1; not shown). All isolates were sensitive to: clindamycin, vancomycin, trimethoprim/sulfamethoxazole, ciprofloxacin, tetracycline, synercid, rifampin, linezolid, daptomycin, and gentamicin (not shown)

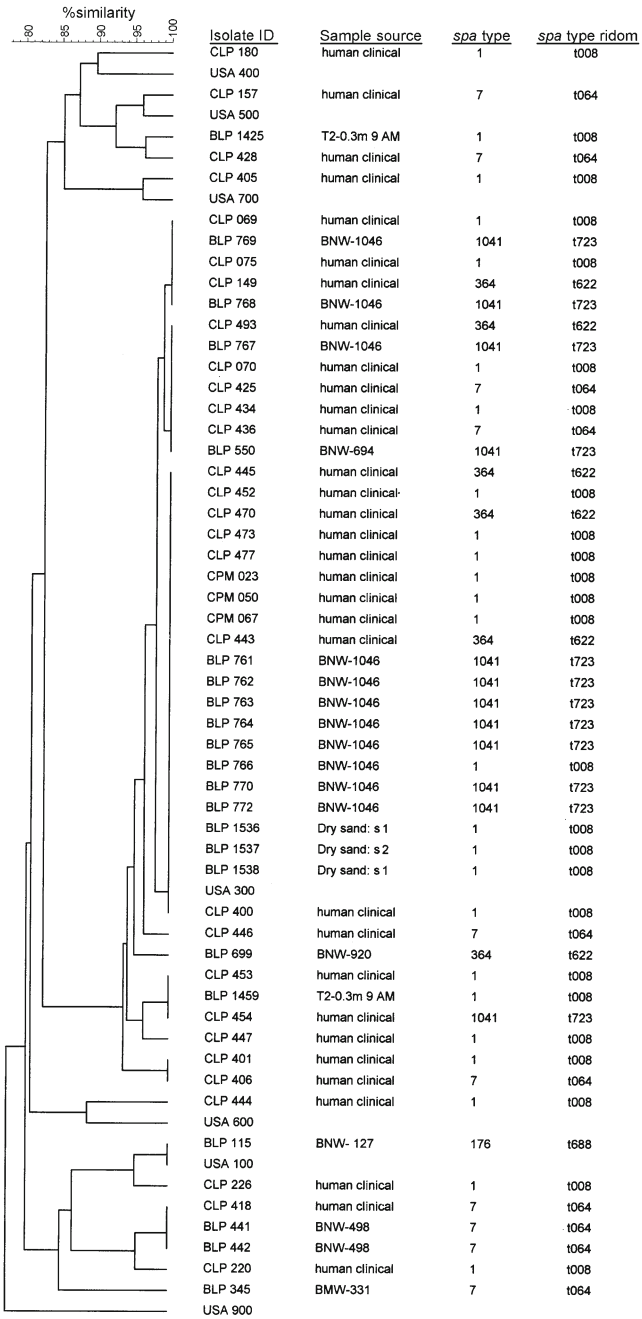

Fig. 4 PFGE-based dendrogram and *spa* types of MRSA study isolates and clinical MRSA isolates obtained from local hospitals or clinics. Bionumerics software was used to generate a dendrogram based on PFGE results as compared to USA prototype controls. Sample sources: *BNW*, bather near water; *T*, transect/ambient water

All other MRSA had genes *eno*, *fnb*B, *fib*, *clf*A, and *clf*B (data not shown). All MRSA had genes *hla*, *hlb*, *hlg*ABC, and *luk*DE (data not shown). Study MRSA were primarily resistant only to the penicillins (e.g., penicillin, ampicillin, oxacillin) and all, except one, to erythromycin.

All sand-isolated MSSA ($n=12$) and 519 of water-associated MSSA (399 from BNW and 120 from ambient water were chosen as representative subsets) were subjected to toxin gene profiling. The prevalence of *eta*, *etb*, *sea*, *seb*, and PVL was determined for representative water-isolated MSSA as compared to *S. aureus* previously recovered from infected skin lesions [47] and is presented in Table 2. A single sand-isolated MSSA carried *sea* (data not shown); all other sand isolates were toxin negative. All MSSA tested had genes *hla*, *hlb*, *hlgABC*, and *lukDE* (data not shown).

Discussion

S. aureus is an organism that can be shed by colonized or infected individuals in many environments including hospitals, gyms, locker rooms, and prisons [10–13]. Although there are increasing reports of MSSA and MRSA associated with marine environments and recreational beaches [24–28, 33–38], none have been associated with reported outbreaks of infections. While it is widely accepted that humans could be a probable source of MSSA and MRSA at recreational beaches, few studies have attempted to investigate a direct association with the presence of humans at a beach and the *S. aureus* isolated from that location. Charoenca and Fujioka reported that total staphylococci in Hawaiian marine waters correlated with the number of bathers [33] and further reported that in a pediatric population with a mean age of 7 years, exposure to this water was associated with a reported skin infection [34]. We previously confirmed that colonized humans shed viable MSSA and MRSA into marine waters [41], and Enns et al. [38] demonstrated over a 10-day period with hourly water collections at this study beach that *S. aureus* levels peaked approximately 4 h after bather peaks.

Table 2 Prevalence of toxin genes in marine MSSA isolates as compared to infecting *S. aureus*

	Source of Bacterial Isolate[a]		
Toxin gene	Bather nearby water % positive (399)	Ambient water % positive (120)	Infecting *S. aureus* [49] % positive
eta	1.5 (6)*	0*	43
etb	0*	1 (1)*	39
sea	15.8 (63)*	1 (1)	3
seb	6.5 (26)**	1 (1)*	13
PVL	<1 (1)*	1 (1)*	11

Values inside the parenthesis indicate number of isolates

eta exfoliative toxin A, *etb* exfoliative toxin B, *sea* staphylococcal enterotoxin A, *seb* staphylococcal enterotoxin B, *PVL* Panton–Valentine leukocidin

* $p<0.005$; ** $p=0.075$

[a] Not shown are 12 isolates from sand. All sand isolates were negative for *eta*, *etb*, *sea*, *seb*, and PVL, except one which was positive for *sea* only

In this study, we sought to evaluate the possible relationship between bather density and the presence of *S. aureus* in the water and at the beach in a subtropical location that does not have a point source for sewage contamination. An earlier study showed that *S. aureus* concentrations in offshore water at the same study site was primarily less than the detection limit of 1 CFU/100 mL when the bather density was very low [39]. This study also showed that approximately 10^5 CFU of *S. aureus* could be released into the water by single bather [39]. The number of bathers and *S. aureus* levels were fairly correlated; however, not as strongly as expected, which was likely due to the large dilution factor in open water unlike the earlier study conducted in a small controlled environment (e.g., a pool). Here, results support that bathers are indeed a potential source of the bacteria by showing that the average number of people in the water during a sampling period was correlated with the daily geometric mean of *S. aureus* in these waters (Fig. 2). The results from this and earlier studies suggest that the bathers in the water are likely a primary source for *S. aureus* and not the shoreline, as has been recently observed for the fecal indicator bacteria, enterococci [60].

Here, we determined that recreational bathers were exposed to *S. aureus* in the water at this beach at a range of 31 % for ambient waters to 37 % [57] when bathers collect their own water samples. However, a concurrent increase in reported illness as a result of this exposure was not evident. The potential for infection by *S. aureus* is impacted by a number of factors in otherwise healthy individuals where these infections are usually limited to the skin. Among these factors are the virulence potential of the bacteria themselves, which in part are associated with the ability of the organisms to express virulence factors. A possible contributing reason for the lack of reported illness despite exposure to these bacteria could be linked to the predominant *S. aureus* population to which the bathers were exposed. The vast majority of the *S. aureus* isolated in this environment were MSSA, likely not capable of expressing skin infection-associated toxins, as was shown by a direct comparison of toxin gene profiles of these organisms to a population of *S. aureus* isolated from infected skin lesions in otherwise healthy individuals [47]. Although any MSSA may be pathogenic, our results indicate that these marine water-related MSSA were not. In addition, only a small percentage of the *S. aureus* isolated during this study (1–3 %) were MRSA.

All the MRSA isolated from multiple sources and multiple times during this study were identical to (86 %) or strongly resembled MRSA clinical isolates from local hospitals or clinics. The majority of the MRSA isolated were indistinguishable from or clonally related to CA-MRSA USA300 by established PFGE criteria [55] and the remaining MRSA clustered with other MRSA known to be responsible for human infection (USA700 and USA100; [61]). These results are in contrast to the characterization of MRSA isolated from other recreational beaches in the Northwestern US where the isolates were similar to multidrug-resistant hospital strains [27] or were primarily associated with animals [26], but in agreement with reports from marine MRSA from Hawaii where CA-MRSA have been reported [62]. This diversity in MRSA populations was likely related to the sources of organisms at these locations, which these results suggest were at least in part due to MRSA-colonized beachgoers.

The lack of corresponding reported illness during this study suggests that although bathers were exposed to *S. aureus*, the majority of these organisms were unlikely to be associated with infection in the average healthy beachgoer. Additionally, exposure to organisms with a greater propensity to cause infection (e.g., CA-MRSA) was in fact quite low. The concentration of the organisms in the water was probably not sufficient to establish an infection in a host with a normal immune system and non-damaged skin. Furthermore, illness was self-reported at 7 days which meant a lack of objective confirmation after a potentially inadequate time period. Finally, the sample size of the BEACHES study was not sufficient to fully explore the relationship between *S. aureus* exposure and reported health effects.

Additional limitations of this study included the fact that bather counts corresponded to a very large area (30,000 m^2) , and that the distance between ambient water sample collection and individual bathers was not known. As a result, the relationship between *S. aureus* levels and bather density could have been confounded by the manner in which bathers were counted. The impact of bather proximity was likely averaged out when the data were aggregated on a daily basis, thereby elucidating relationships between average daily bather density and the daily geometric mean of *S. aureus*.

Characterization of CA-MRSA in the environment and possible sources of exposure and infection for the general population should be studied, and potential risk of illness related to these environmental exposures should be quantified and reduced, if possible. The results of this study suggest that bather load in the water (and potentially the person load on the beach) contributes to the number of organisms in the water, and subsequently to the risk of exposure to potentially pathogenic bacteria. Additional sources of MSSA and MRSA to the beach environment could also exist but were beyond the scope of this study to determine. Beachgoers are likely exposed to a combination of the organisms including those they are colonized with themselves and shed into the water, those shed by other beachgoers, and potentially to *S. aureus* persisting and possibly growing in the environment [41].

Our data support that each recreational beach environment will be different based upon the population of people,

number of colonizing organisms, and other sources of microorganisms contributing to that environment. It is also not clear at this time what the potential is for the marine water, sand, flora, and/or fauna to contribute to the microorganisms or to serve as reservoirs for these bacteria.

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