

Virulence and Antibiotic Resistance Gene Combinations among *Staphylococcus aureus* Isolates from Coastal Waters of Oahu, Hawaii

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ABSTRACT

Staphylococcus aureus is often found passively colonizing human skin and nasal passages. As an opportunistic pathogen, the bacterium can also cause infections that vary widely in their severity and in their susceptibility to antibiotic treatment. This variability is caused by differences in gene content among strains. One source of exposure to novel, and potentially more virulent, strains of *S. aureus* is recreational swimming areas, since *S. aureus* is readily shed from humans into water. The standard measure of *S. aureus* contamination in recreational waters is the concentration of colony forming units, but this method does not discriminate among strains of differing virulence. As a first step toward characterizing the diverse strains of *S. aureus* to which beachgoers may be exposed, sixteen strains were isolated from seawater around Oahu and tested for the presence of eight genes associated with either virulence (*tst*, *cna*, *ica*, *hlg[B and C]*, *sea*, and *sdrE*) or antibiotic resistance (*mecA*, *femA*) using the polymerase chain reaction and gene-specific primers. The maximum number of these genes observed in any single isolate was six and the minimum was two with a total of nine different gene combinations observed among the sixteen isolates. The results show that the virulence gene content of *S. aureus* strains isolated from coastal waters varies widely. Further characterization of the diversity of *S. aureus* in the environment could, therefore, contribute to a better understanding of the epidemiology of community-acquired *S. aureus* infections and lead to improved measures of the exposure risk for swimmers in recreational waters.

INTRODUCTION

Staphylococcus aureus is an opportunistic human pathogen capable of causing a wide variety of diseases including septicemia, pneumonia, wound sepsis, septic arthritis, osteomyelitis, food poisoning, and toxic shock syndrome (Boyd and Brüssow 2002; Moore and Lindsay 2001). Most people are passive carriers of *S. aureus*, which is often found colonizing the nasal passages (Kluytmans et al. 1997), and in some cases, an infection may arise from self-inoculation of a wound. *S. aureus* infections have been a major problem in hospitals for decades, but the incidence of community-acquired infections has also been increasing (Chambers 2001).

The type as well as severity of an *S. aureus* infection and its response to antibiotic treatment are dictated by the specific suite of virulence- and antibiotic resistance-associated genes carried by the strain of *S. aureus* causing the infection (Peacock et al. 2002). There are over 40 virulence-associated genes identified among various strains of *S. aureus*, many of which are encoded by mobile genetic elements. Until recently, methicillin was one of the few remaining antibiotics effective against *S. aureus*, but the emergence and spread of methicillin-resistant *S. aureus*

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(MRSA) has severely decreased the usefulness of this antibiotic. While this problem was initially confined to clinical settings (Chambers 2001), the frequency of community-acquired MRSA is becoming more common and some of these community-acquired strains are particularly virulent (Baba et al. 2002).

A correlation between seawater exposure and *S. aureus* infection rates (Charoenca and Fujioka 1995) suggests that the oceans may be one pathway by which community-acquired infections are transmitted. *S. aureus* is readily shed from people while swimming or engaging in other water-related activities (Charoenca and Fujioka 1993) and high counts of *S. aureus* in recreational waters is often considered a risk factor for contracting many diseases that affect areas such as the skin, eyes, and ears (Gabutti et al. 2000). Although *S. aureus* is routinely found in the waters surrounding the island of Oahu in the state of Hawaii (Charoenca and Fujioka 1995; Charoenca and Fujioka 1993), nothing is known yet about the distribution of virulence genes among the various strains recovered from environmental sources. Most investigations of genetic diversity have focused on isolates collected directly from healthy or diseased humans (Diep et al. 2004; Monk et al. 2004; Nashev et al. 2004). To better evaluate risk, it would be helpful to determine not only total counts of *S. aureus* in recreational waters, but also the number and type of virulence-associated genes that they carry.

Recent studies suggest that adaptations to the external environment (Wilson and Salyers 2003) and lateral gene transfer among strains (Boyd and Brüssow 2002; Fitzgerald et al. 2001) may play important roles in the evolution of pathogens. Knowledge of the genetic diversity of *S. aureus* in the environment could, therefore, shed light on the emergence and spread of novel pathogenic strains. In this preliminary study, *S. aureus* strains were isolated from several beaches around Oahu and from a canal in downtown Waikiki for partial genetic characterization. DNA from each isolate was then screened by the polymerase chain reaction (PCR) for the presence or absence of six virulence-associated genes and two genes associated with methicillin resistance

METHODS AND MATERIALS

Sample Collection

Water samples were collected by hand in acid-washed polycarbonate, or autoclaved polypropylene, containers from Kailua Beach on the eastern shore of Oahu, from Kuhio, Sans Souci, and Ala Moana beaches on the southern shore of Oahu, and from the Ala Wai canal, which runs inland along the northern edge of Waikiki. For sampling at beaches, the collector waded into knee- to waist-deep water and submerged the collection vessel in the vicinity of swimmers. The vessel was kept in front of the collector who moved forward through the water as the vessel filled. At the Ala Wai canal, a bottle was submerged from a boat dock without requiring contact of the collector with the water. Bottles were immediately capped and handled aseptically through the isolation process.

*Isolation and Cultivation of *S. aureus**

Bacteria were collected from seawater samples by filtration onto sterile, 47 mm-diameter, 0.45 um pore-size mixed cellulose ester filters (Pall Corporation, East Hills, New York, or Millipore Corporation, Billerica, Massachusetts). The filters were overlaid onto the selective medium CHROMagar™ Staph aureus (CSA; CHROMagar Microbiology, Paris, France) or onto CSA supplemented with oxacillin (4µg ml⁻¹), sodium azide (0.0035%), or sodium azide plus glycine

(12 g l⁻¹). Alternatively, filters were incubated in Nutrient Broth with oxacillin to enrich for *S. aureus* prior to plating on solid media as above. Putative *S. aureus* colonies were picked from the filters and purified by serially picking and streaking individual colonies on fresh CSA or tryptic soy agar plates. Isolates were screened for methicillin resistance by plating on oxacillin-containing media. Putative MRSA isolates were confirmed using the disk diffusion test and a PBP2' agglutination assay.

DNA Purification

Each purified *S. aureus* isolate was grown to stationary phase in tryptic soy broth. Cells in 3 ml of culture were then pelleted by centrifugation for 10 minutes at 10,000 × g then resuspended in 25 µl of TER Buffer (10 mM Tris-Cl, 1mM EDTA, 0.1%SDS, 100 µg ml⁻¹ RNase A). Resuspended cells were extracted using a potassium ethyl xanthogenate protocol (Tillet and Neilan 2000) combined with bead beating. XS buffer was prepared immediately before use by combining 0.1 g of potassium ethyl xanthogenate (Sigma-Aldrich, Corp., Saint Louis Missouri), 1 ml of 1 M Tris at pH 7.4, 0.4 ml of 0.5 M EDTA at pH 8, 1 ml of 10% SDS, 2 ml of 4 M ammonium acetate, and 5.6 ml of ultrapure water (Nanopure Diamond, Barnstead International, Dubuque, Iowa). XS buffer (750 µl) was added to the resuspended pellets and the mixture transferred to 2 ml-capacity, screw-cap microcentrifuge tubes containing 0.5 g of 0.1 mm zirconium beads (Biospec Products, Inc. Bartlesville, Oklahoma). Samples were shaken in a FastPrep™ instrument (Q-Biogene, Carlsbad, California) for 30 s at a setting of 6 m s⁻¹. All samples were then incubated at 70 °C for 1 hour followed by brief vortex mixing and incubation on ice for 30 minutes. The samples were centrifuged at 14,000 rpm for 10 minutes at 4°C to pellet precipitated protein-SDS complexes and the supernatants containing the extracted DNA were transferred to fresh tubes.

Precipitating DNA

GlycoBlue™ (Ambion, Inc.) was added to each of the supernatants at a final concentration of 50 µg ml⁻¹ to aid in precipitation. An equal volume of isopropanol was added, the samples were then mixed by several inversions of the tube, incubated for 10 min at room temperature, then centrifuged at 8,000 rpm for 30 minutes at 4° C. The supernatant was discarded and the pellet was washed by adding 1 ml of 70% ethanol followed by gentle mixing to rinse the entire inner surface of the tube. The pellet was centrifuged as before, but for only 10 min, then the supernatant was removed and the pellets were allowed to air dry completely. All pellets were resuspended in 50 µl of TE buffer (100mM of Tris-Cl and 10mM EDTA).

Quantifying Extracted DNA

DNA extracts were analyzed qualitatively by electrophoresis in a 0.8% agarose gel. DNA in the gel was stained with ethidium bromide (0.005%, w/v) and visualized on a UV transilluminator. Images of gels were captured with a digital gel documentation system (Gel Doc-It, U). DNA yields were also quantified by fluorometry using a DNA Quantification kit (Quant-iT DNA assay, Molecular Probes, Inc., Eugene Oregon) and a filter-based fluorometer (TD700, Turner Designs).

PCR amplification

Six virulence-associated genes (*cna*, *tst*, *sdrE*, *sea*, *hlg[B and C]*, and *ica*) and two genes associated with antibiotic resistance (*femA*, *mecA*) were amplified from DNA extracts using the Expand High Fidelity PCR kit (Roche, Indianapolis, Indiana) using primers and thermal cycling

protocols specific to each gene (Table1). The reaction contained 100 μmol primers and 0.5 μl template (6 to 31 ng) in a final reaction volume of 50 μl.

Table 1. Genes targeted and the PCR conditions used for amplification. All PCR reactions began with a 95° C denaturation for 5 minutes and were terminated with 72° C extension for 7 minutes and a 4° C hold. Product length for *cna* may vary by multiples of 561 bp as a result of sequence repeats of the B region. Due to sequence similarities, the primers for *hlg* will co-amplify *hlgB* and *hlgC*.

Gene (role)	Primer Sequences (F)orward and (R)everse	PCR Cycling Conditions	Product Length (bp)	Ref.
<i>cna</i> (collagen adhesin)	F: AGTGGTTACTAATACTG R: CAGGATAGATTGGTTTA	1 min 95° C 1 min 50° C 2 min 72° C	744	(Peacock et al. 2002)
<i>hlg</i> (hemolysin)	F: GCCAATCCGTTATTAGAAAATGC R: CCATAGACGTAGCAACGGAT	1 min 95° C 1 min 55° C 1 min 72° C	937	(Lina et al. 1999)
<i>icaA</i> (intracellular adhesin)	F: GATTATGTAATGTGCTTGGA R: ACTACTGCTGCCGTTAATAAT	1 min 95° C 1.5 min 60° C 1.5 min 72° C	770	(Peacock et al. 2002)
<i>sdrE</i> (putative adhesin)	F: CAGTAAATGTGTCAAAGA R: TTGACTACCAGCTATATC	1 min 95° C 2 min 55° C 1 min 72° C	767	(Peacock et al. 2002)
<i>sea</i> (enterotoxin)	F: GGATATTGTTGATAAATATAAAGGGAAAAAAG R: GTTAATCGTTTTATTATCTCTATATATTCCTTAATAGT	1 min 95° C 1 min 55° C 1 min 72° C	439	(Sergeev et al. 2004)
<i>tst</i> (toxic shock toxin)	F: ATGGCAGCATCAGCTTGATA R: TTTCCAATAACCACCCGTTT	2 min 95° C 2 min 55° C 1 min 72° C	350	(McLaughlin et al. 2000)
<i>femA</i> (methicillin resistance)	F: AACAGCTAAAGAGTTTGGTGCC R: CATCACGATCAGCAAAAGCT	1 min 95° C 1.5 min 60° C 2 min 72° C	647	This study
<i>mecA</i> (methicillin resistance)	F: CGGTAACATTGATCGCAACG R: TTTGCCAACCTTTACCATCG	45 sec 95° C 1 min 60° C 2 min 72° C	985	This study

Cloning and Sequencing of Virulence Genes

A portion of each PCR product (2 μl) was ligated into the pCR 2.4-TOPO vector and transformed into a TOP10 chemically competent *E. coli* using the TOPO TA[®] Cloning kit (Invitrogen, Carlsbad California). Following transformation, 20 and 40 μl of the transformation mixture were spread onto LB agar plates which had been previously supplemented by spreading 100 μl of 2% x-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) per plate. Plates were incubated at 37°C overnight then two colonies of each type of clone were selected through blue/white screening and transferred to 2-ml tubes with air-permeable caps (Eppendorf[®] LidBAC, Brinkmann Instruments, Inc., Westbury, New York) containing 1.5 ml of LB broth with kanamycin (Sigma St. Louis, Missouri). Tubes were incubated in a shaking incubator (VorTemp Microtube Incubator Shaker, Midwest Scientific, Valley Park, Missouri) at 37°C for 24 hours. After incubation, cells were pelleted by centrifugation for 10 minutes at 10,000 ´ g at room temperature. Plasmid DNA was purified using a QIAprep Spin Miniprep kit per manufacturer’s instructions (Qiagen). To verify that the selected clones contained an insert of the expected size, 2 μl of this solution was digested with the restriction enzyme EcoR1 and the product size was analyzed by gel electrophoresis on a 2% gel. To verify that the cloned products were the

targeted virulence genes, one clone representing each gene was sequenced using CEQ Dye Terminator Cycle Sequencing with Quick Start kit (Beckman Coulter, Inc. Fullerton, California). The sequencing reactions were analyzed on a model CEQ 200XL sequencer (Beckman Coulter, Inc).

RESULTS

Each of the genes could be successfully amplified by PCR from at least one of the isolates (Fig. 1), but no isolate was positive for all eight of the genes. Overall, among the 16 isolates, nine different combinations of the targeted genes were found (Table 2). The most commonly occurring gene, *sea*, was found in all but one of the isolates tested. In contrast, *cna* and *tst* were found in only one isolate each. One genotype contained as few as two of the eight genes while eight genotypes contained six.

Eight *S. aureus* isolates previously determined to be resistant to methicillin were shown to have the expected methicillin resistance-associated genes *mecA* and *femA*. However, five of eight isolates that were sensitive to methicillin by the disk diffusion test were also found to have both the *mecA* and *femA* genes.

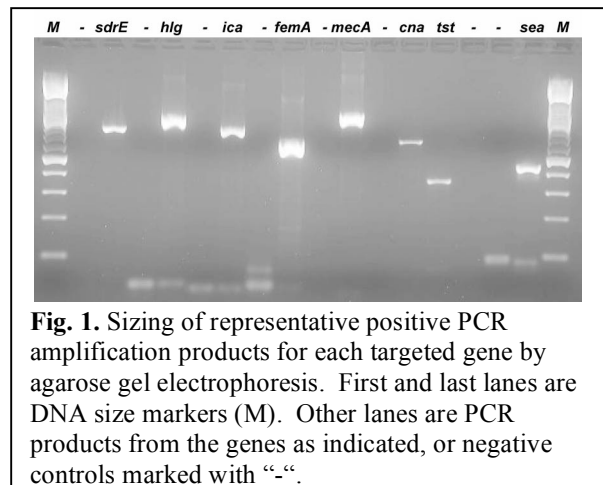


Fig. 1. Sizing of representative positive PCR amplification products for each targeted gene by agarose gel electrophoresis. First and last lanes are DNA size markers (M). Other lanes are PCR products from the genes as indicated, or negative controls marked with “-”.

Table 2. The distribution of genes associated with antibiotic resistance and virulence among *S. aureus* strains isolated from the Ala Wai canal in Waikiki and various beaches around Oahu.

Sequence		Methicillin Resistance		Virulence-Associated Genes					
		Phenotype (+/-)	Genes (<i>mecA</i> <i>femA</i>)	<i>cna</i>	<i>hlg</i>	<i>ica</i>	<i>sdrE</i>	<i>sea</i>	<i>tst</i>
1	Kuhio	+	• •		• •	• •	• •	• •	
	Kuhio	+	• •		• •	• •	• •	• •	
	Sans Souci	+	• •		• •	• •	• •	• •	
	Sans Souci	+	• •		• •	• •	• •	• •	
	Ala Wai	-	• •		• •	• •	• •	• •	
	Ala Wai	-	• •		• •	• •	• •	• •	
	Ala Moana	-	• •		• •	• •	• •	• •	
2	Ala Moana	+	• •		• •			• •	
3	Sans Souci	+	• •		• •	• •	• •		
4	Sans Souci	+	• •		• •		• •	• •	
5	Kuhio	+	• •			• •		• •	
6	Kailua	-	• •		• •	• •		• •	• •
7	Ala Moana	-	• •		• •				• •
8	Ala Wai	-	• •		• •	• •	• •	• •	
	Ala Wai	-	• •		• •	• •	• •	• •	
9	Ala Wai	-				• •		• •	

DISCUSSION

Although there are many known virulence-associated genes in *S. aureus*, this preliminary study focused on only a small subset. Some of these (*cna*, *hlg*, *icaA*, *sdrE*) were chosen because they were among those found to be more common among invasive isolates (Peacock et al. 2002). Assays for staphylococcal enterotoxin A (*sea*) and toxic shock toxin (*tst*) were included, because they can be transferred laterally among strains via bacteriophage infection (Ruzin et al. 2001; Betley and Mekalanos 1985). The number of different combinations in which the eight selected genes were observed among the sixteen isolates indicates that genetic diversity of *S. aureus* in coastal waters of Oahu is high. There was no obvious correlation between the combination of genes found in an isolate and the location from which it was derived suggesting that the observed diversity is not restricted by geography. Few isolates from each site were analyzed, however, and some pattern to the distribution of genotypes may appear as additional isolates from more sites are analyzed.

The virulence genes *mecA* and *femA* were examined because they are believed to be major contributors to methicillin resistance (Maidhof et al. 1991; Kuhl et al. 1978). While *mecA* and *femA* genes were found in all eight of the methicillin-resistant strains, they were also found in five methicillin-susceptible strains. In fact, the same combination of the eight tested genes was found in both methicillin-susceptible and a methicillin-resistant strains. The PCR-based assays used in this study only indicate presence or absence of a gene. The discrepancy between the resistance assays and gene content could therefore be due to differences in the expression of *mecA* and *femA* among strains. Alternatively, there may be additional factors essential for methicillin resistance that were not examined in this study.

CONCLUSIONS

Both the number and the specific combinations of virulence- and antibiotic resistance-associated genes in *S. aureus* isolates from the coastal waters of Oahu were found to vary widely. This suggests that more accurate assessments of health risks to bathers may require not only total colony-forming units of *S. aureus*, but also an assessment of the virulence gene content of those colonies. In addition, the detection of both *mecA* and *femA* in methicillin-sensitive isolates suggests that presence of these two genes cannot be used alone as diagnostic indicators for methicillin resistance. Further work to determine accurate molecular indicators of antibiotic resistance will be useful for rapid non-cultivation based diagnostic assays.

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