Staphylococcus aureus and methicillin-resistant *S. aureus* (MRSA) at ambient freshwater beaches

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) are a threat to human health worldwide, and although detected at marine beaches, they have been largely unstudied at freshwater beaches. Genes indicating *S. aureus* (SA; *femA*) and methicillin resistance (*mecA*) were detected at 11 and 12 of 13 US Great Lakes beaches and in 18% or 27% of 287 recreational water samples, respectively. Eight beaches had *mecA* + *femA* (potential MRSA) detections. During an intensive study, higher bather numbers, staphylococci concentrations, and *femA* detections were found in samples collected after noon than before noon. Local population density, beach cloud cover, and beach wave height were significantly correlated with SA or MRSA detection frequency. The Panton-Valentine leukocidin gene, associated with community-acquired MRSA, was detected in 12 out of 27 potential MRSA samples. The *femA* gene was detected less frequently at beaches that met US enterococci criteria or EU enterococci 'excellent' recreational water quality, but was not related to *Escherichia coli*-defined criteria. *Escherichia coli* is often the only indicator used to determine water quality at US beaches, given the economic and healthcare burden that can be associated with infections caused by SA and MRSA, monitoring of recreational waters for non-fecal bacteria such as staphylococci and/or SA may be warranted.

Key words | bathing water quality, CA-MRSA, methicillin-resistant *Staphylococcus aureus*, recreational water, *Staphylococcus aureus*

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INTRODUCTION

Approximately 20% of the 5,920 km of US Great Lakes coastline is a designated swimming beach, and the number of beaches per 10 km of US Great Lakes coastline (ca. 1.5) is similar to that of marine coastal beaches in the European Union (EEA 2013). Beaches in many parts of the world are monitored for bacteria that may indicate bathing water pollution from fecal sources such as sewage: *Escherichia coli* and enterococci (ENT) (WHO 2003; USEPA 2012; EEA 2013). Nevertheless, non-fecal bacteria such as *Staphylococcus aureus* (SA) are a recognized health hazard in swimming pools (WHO 2006) and at beaches (WHO 2003), where they are assumed to be largely derived from bather shedding (up to 10^5–10^6 colony forming units (CFU) per person) (Elmir *et al.* 2007; Plano *et al.* 2011). SA is an opportunistic pathogen causing skin and soft-tissue infections, sometimes severe or life-threatening, especially when resistant to methicillin (MRSA) (David & Daum 2010). Recent studies have detected MRSA in marine waters (Viau *et al.* 2005; Tolba *et al.* 2008; Goodwin & Pobuda 2009; Soge *et al.* 2009; Roberts *et al.* 2013), but there is limited information regarding the occurrence of MRSA at freshwater beaches.

Initially, MRSA isolates were only identified in healthcare settings (HA-MRSA), but have now emerged as widespread community-acquired (CA-MRSA) infections (David & Daum 2010). HA-MRSA and CA-MRSA strains

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are genetically different (David & Daum 2010). Nearly all MRSA strains contain the staphylococcal cassette chromosome *mec* (SCC*mec*) element (types I–VIII), which carries the methicillin resistance determinant *mecA* gene (Katayama *et al.* 2000). SCC*mec* types I–III are typically associated with HA-MRSA whereas types IV and V are associated with CA-MRSA (David & Daum 2010). In addition, many CA-MRSA strains carry the Panton-Valentine leukocidin (PVL) virulence genes, differentiating them from HA-MRSA (Boyle-Vavra & Daum 2006).

CA-MRSA infections in the USA have become increasingly prevalent since the early 1990s; the prevalence in European countries is comparatively low, but increasing (Otter & French 2010). Rubio-Terres *et al.* (2010) reported longer hospital stays and more intensive care admissions associated with CA-MRSA (as opposed to methicillin susceptible SA) in Spain. Bothwell *et al.* (2007) reported that, over 5 years, CA-MRSA head or neck infections rose from 21 to 64% in a coastal community in Hawaii, and specifically noted the role of swimming exposure as a potential factor.

The objectives of this study were to determine the occurrence of SA and MRSA at freshwater beaches, determine environmental factors that may be related to their occurrence, and evaluate occurrence in terms of US and EU recreational water-quality criteria (RWQC) based on *E. coli* or ENT.

METHODS

Study locations and sampling

Characteristics for 12 of the 13 beaches studied are reported in Haack *et al.* (2013). One additional site was added for the study: Lake Michigan site 6, located in Michigan with 44% urban, 27% forest, 14% agriculture, 2% open water, and 12% other land cover. Beach water was sampled 17–27 times per beach from June to September 2010; water was shipped overnight on ice and processed the following day. *Escherichia coli* concentrations and sanitary survey conditions for the beaches on each sample collection date were provided by local beach managers. Enterococci concentrations were determined on received samples using membrane filtration on mEI agar (USEPA 2006). An intensive study was conducted at the Lake Michigan 5 site on 7, 13, and 28 August 2010. Water samples were collected at six sampling locations on the beach at 2-h intervals to determine if *Staphylococcus* concentrations changed over the course of the day.

Staphylococcus culturing and preservation

Staphylococci concentrations were evaluated on 100 mL beach-water samples using standard membrane filtration on Baird Parker (BP) agar (American Water Works Association & Water Environment Federation 1998). Filters with greater than 300 colonies were not countable and concentrations are reported as >300 CFU/100 mL. Growth from these filters was collected by 15 min agitation in 10 mL phosphate-buffered saline (PBS), followed by centrifugation and pellet resuspension in 1 mL of 20% glycerol/0.5 × PBS (hereafter – BP glycerol stock), then stored at −70 °C until analysis. For the intensive study at the Lake Michigan 5 site, 100, 10, and 1 mL of the samples were filtered to more accurately determine staphylococci concentrations.

Polymerase chain reaction

DNA was extracted from 400 μL of BP glycerol stock using the QIAamp® DNA Mini Kit following the manufacturer's protocol for isolation of genomic DNA from Gram-positive bacteria (Qiagen, Valencia, CA, USA). DNA concentrations and purity were determined with a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). Template DNA was stored at −20 °C until needed for polymerase chain reaction (PCR).

Multiplex PCR was used to detect the *mecA* (methicillin resistance) and *femA* (SA) genes as described in Haack *et al.* (2013). Analysis of the *luks-PV* (hereafter, PVL) gene that encodes for Panton-Valentine leukocidin was performed following Reischl *et al.* (2000). Single PCR reactions were performed to distinguish the five most commonly detected SCC*mec* genetic elements, SCC*mec* I–V, following Japoni *et al.* (2011). PCR products were visualized using Blue/Orange Loading Dye, 6× (Promega) on 2.2% FlashGel DNA cassettes (Lonza, Walkersville, MD, USA) for 10 min at 170 V. PCR positive and negative controls (no template reactions) were included for approximately every 20 samples.

MRSA isolation and identification

Aliquots of beach-water BP glycerol stocks positive for the *femA* gene were inoculated to Mueller Hinton Broth (MHB) with 4 mg/L cefoxitin (CEF) (Fang & Hedin 2006), and incubated overnight (24 h) at 37 °C. The subsequent inoculum was plated onto Mannitol Salt Agar + 4 mg/L CEF, and incubated for 48 h at 37 °C. Colonies with yellow pigment or yellow media around colony that is typical MRSA morphology were incubated overnight in MHB + CEF. Growth was preserved and DNA extracted as described above. DNA for the selected isolates was amplified using 16S rDNA primers 27F and 1389R (Hongoh *et al.* 2003), prepared according to Michigan State University (East Lansing, MI, USA) Research Technology Support Facility (http://rtsf.natsci.msu.edu/) specifications, and sent to the facility for 16S rDNA sequencing. DNA sequences were aligned using the Ribosomal Database (RDP; http://rdp.cme.msu.edu/), Classifier Version 2.5. Hierarchical taxa assignment was based on the RDP naïve Bayesian rRNA Classifier (Wang *et al.* 2007).

Relation to environmental factors

For all sites but Lake Michigan site 6 (not all environmental data were available), concentrations of staphylococci and frequency of occurrence of *mecA*, *femA*, and *mecA* + *femA* in beach-water samples were correlated to 55 variables describing beach characteristics, sanitary conditions, weather, and hydrometeorological factors. These variables are described in more detail by Haack *et al.* (2013). For samples having a reported value of >300 CFU staphylococci/100 mL, the maximum value of 300 was used in statistical calculations. Statistical analyses were performed in Systat 13 (Systat Software, Chicago, IL, USA) or TIBCO Spotfire S+ (TIBCO Software, Boston, MA, USA).

RESULTS

Quality assurance and control

Of 11 field blank samples (sterile bottles filled with sterile PBS in the field and shipped with regular samples), three had detectable levels of staphylococci; the highest concentration measured was 8 CFU/100 mL. No *mecA* or *femA* genes were detected in any field blank sample. The relative standard deviation of staphylococci concentration (standard deviation/average value × 100) for nine paired field replicate samples ranged from less than 1 to 9%. Both samples in the nine pairs were also analyzed for the presence or absence of *mecA* and *femA* genes. The *mecA* results agreed in all nine pairs (both samples were either positive or negative in each pair); the detection of *femA* agreed in eight pairs.

Staphylococci concentrations

Staphylococci concentrations determined for 278 of the 287 water samples ranged from <1 to >300 CFU/100 mL (method quantification levels), with a median concentration of 65 CFU/100 mL. Staphylococci concentrations were not determined for nine samples due to overgrowth or smearing of colonies on the plate preventing enumeration. These samples were, however, preserved and analyzed for the presence of *mecA* and *femA* genes. The variability in staphylococci concentration between beaches and even on the same beach throughout the sampling season is shown in Figure 1. Median staphylococci concentrations range from 10 CFU/100 mL at Lake Huron 1 to greater than 300 CFU/100 mL at the Lake Michigan 2 site. Only four samples had less than 1 CFU/100 mL and all four were from the Lake Huron 1 site. In contrast, 68 samples were greater than the maximum level of quantification of 300 CFU/100 mL. Twenty-one percent of the staphylococci concentrations were above the quantification limit of 300 CFU/100 mL; therefore, the average and ranges for the data cannot be determined.

Relation between staphylococci and indicator bacteria concentrations

Staphylococci concentrations were compared to US and EU *E. coli* and ENT RWQC. US RWQC include a geometric mean (GM) of 126 *E. coli* or 35 ENT CFU/100 mL and statistical threshold value (STV; no more than 10% exceedance) of 410 for *E. coli* or 130 for ENT (USEPA 2012). The GM and STV values are based on any 30-day interval, and both must be met to be in compliance. For this study, we used the reported *E. coli* or measured ENT concentrations from all

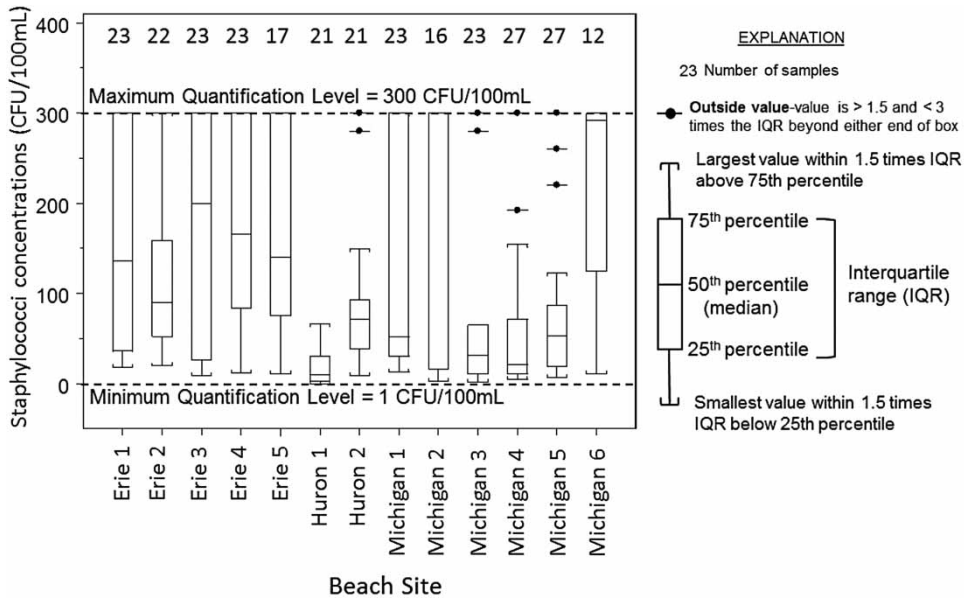

Figure 1 | Box plot of staphylococci concentrations determined at each study beach throughout the sampling season. Samples greater than 300 CFU/100 mL are shown as 300 CFU/mL.

samples for each beach to calculate the GM and STV. EU Directive 2006/7/EC (EU 2006) defines four classifications of water quality based on a 4-year trend, with a minimum of four yearly samples (16 samples). Although only representing 1 year, all but one studied beach had 16 or more samples in our study. Therefore, EU water-quality classifications were assigned using the total number of samples per beach (Tables 1 and 2). Notably, US and EU ENT RWQC were more difficult to achieve than *E. coli* RWQC for these Great Lakes beaches (Tables 1 and 2). For the purpose of this study, EU criteria for coastal and inland waters were presented, because the large size and lengthy coastlines in the US Great Lakes are similar to European coastal beaches; however, the Great Lakes are fresh water (similar to European inland waters), which may also influence bacterial survival. Lower staphylococci concentrations were found at beaches that met US RWQC or had excellent EU coastal *E. coli* and ENT quality (Figure 2), and the difference was significant for both *E. coli* and ENT under both US and EU criteria (Mann–Whitney U test; all $P < 0.002$).

The beach action value (BAV) is an optional US *E. coli* (235 CFU/100 mL) or ENT (70 CFU/100 mL) recommendation that can be used for monitoring and notification programs based on daily samples (USEPA 2012); however, beach managers do not use ENT to assess water quality at any Great Lakes beaches. This study predated the publication of the BAV recommendation, and a single-sample standard of 235 CFU/100 mL for *E. coli* was used at most Great Lakes beaches at the time of this study. For beaches in Michigan, the Michigan Department of Environmental Quality standard of 300 CFU *E. coli*/100 mL as a GM of three concurrent samples was used. Of 13 beaches sampled, all but three had at least one exceedance of a beach BAV for *E. coli* and all but one had at least one exceedance of the BAV for ENT (Tables 1 and 2). In the majority of cases, the single-sample staphylococci concentration was greater on the day when the BAV was exceeded, as opposed to when it was met (data not shown).

Detection of SA and methicillin resistance genes and relation to beach-water-quality criteria

The *femA* gene was detected at 11 of the 13 beaches (Table 3). Of 287 beach samples analyzed, *femA* was detected in 51 samples (18%), and of those 51, 27 (53%; 9% of all samples) were also positive for *mecA*. Using Spearman's rank correlation, the frequency of detection of *femA*, *mecA*, and *femA* + *mecA* was evaluated against log10 of the GM concentration of staphylococci for each beach, but no

Table 1 | Staphylococci concentrations (CFU/100 mL) compared to US and EU recreational water-quality criteria (RWQC) and the US beach action value (BAV) for *E. coli* at Great Lakes beaches

Beach	Total number of samples for *E. coli*[a]	Beach met the US RWQC[b]	EU *E. coli* coastal quality status[c]	EU *E. coli* inland lakes quality status[d]	% of samples that exceed state's BAV[e]	Median staphylococci concentration	Median staphylococci concentration when BAV met	Median staphylococci concentration when BAV exceeded
Lake Erie 1 (OH)	23	No	Poor	Poor	35	136	68	> 300
Lake Erie 2 (OH)	23	No	Excellent	Excellent	22	90	59	> 300
Lake Erie 3 (NY)	22	Yes	Good	Excellent	14	200	188	> 300
Lake Erie 4 (NY)	23	Yes	Good	Excellent	13	166	124	> 300
Lake Erie 5 (NY)	17	No	Poor	Poor	29	140	92	> 300
Lake Huron 1 (MI)	21	Yes	Excellent	Excellent	0	10	> 300	NA[f]
Lake Huron 2 (MI)	21	No	Good	Excellent	14	71	70	> 300
Lake Michigan 1 (WI)	20	No	Poor	Poor	50	52	30	> 300
Lake Michigan 2 (WI)	23	Yes	Excellent	Excellent	4	> 300	> 300	> 300[g]
Lake Michigan 3 (WI)	22	Yes	Excellent	Excellent	18	31	26	45
Lake Michigan 4 (MI)	26	Yes	Excellent	Excellent	0	21	45	NA
Lake Michigan 5 (MI)	26	Yes	Excellent	Excellent	0	53	51	NA
Lake Michigan 6 (MI)	12	Yes	Excellent	Excellent	8	292	284	> 300
Total	279				15		58	> 300

[a]Only includes samples with associated *E. coli* concentrations. *Escherichia coli* concentration was not determined for a small number of samples included in this study.
[b]United States Environmental Protection Agency, 2012 recreational water-quality criteria (USEPA 2012). Yes = beach met both the geometric mean (GM) of 126 CFU/100 mL and statistical threshold value (STV) of 410 CFU/100 mL values for *E. coli* based on the reported *E. coli* values over the course of the study. No = beach failed one or both criteria.
[c]Directive 2006/7/EC of the European Parliament and of the Council of 15 February 2006 concerning the management of bathing water quality and repealing Directive 76/160/EEC. Official Journal of the European Union L 64/37, 2006. Excellent: <250 CFU/100 mL based on a 95th percentile evaluation of 16 samples over 4 years; good: <500 CFU/100 mL based on a 95th percentile evaluation of 16 samples over 4 years; sufficient: <500 CFU/100 mL based on a 90th percentile evaluation of 16 samples over 4 years; and poor: does not meet 'sufficient'.
[d]Directive 2006/7/EC of the European Parliament and of the Council of 15 February 2006 concerning the management of bathing water quality and repealing Directive 76/160/EEC. Official Journal of the European Union L 64/37, 2006. Excellent: <500 CFU/100 mL based on a 95th percentile evaluation of 16 samples over 4 years; good: <1,000 CFU/100 mL based on a 95th percentile evaluation of 16 samples over 4 years; sufficient: <900 CFU/100 mL based on a 90th percentile evaluation of 16 samples over 4 years; and poor: does not meet 'sufficient'.
[e]Beach action value (BAV), United States Environmental Protection Agency, 2012 recreational water-quality criteria (USEPA 2012); 235 CFU/100 mL.
[f]NA, beach did not have any sample that exceeded BAV; therefore, no value could be determined.
[g]Seven out of the 23 samples were not quantifiable.

Table 2 | Staphylococci concentrations (CFU/100 mL) compared to US and EU recreational water-quality criteria (RWQC) and the US beach action value (BAV) for enterococci at Great Lakes beaches

Beach	Total number of samples for enterococci	Beach met the US RWQC[a]	EU enterococci coastal quality status[b]	EU enterococci inland lakes quality status[c]	% of samples that exceed state's BAV[d]	Median staphylococci concentration	Median staphylococci concentration when BAV met	Median staphylococci concentration when BAV exceeded
Lake Erie 1 (OH)	23	No	Poor	Poor	35	136	72	> 300
Lake Erie 2 (OH)	23	Yes	Good	Excellent	13	90	65	> 300
Lake Erie 3 (NY)	23	No	Sufficient	Good	17	200	90	> 300
Lake Erie 4 (NY)	23	No	Sufficient	Sufficient	17	166	123	> 300
Lake Erie 5 (NY)	17	No	Poor	Poor	35	140	90	> 300
Lake Huron 1 (MI)	21	Yes	Excellent	Excellent	0	10	10	NA[e]
Lake Huron 2 (MI)	21	No	Poor	Sufficient	67	71	38	> 300
Lake Michigan 1 (WI)	24	No	Poor	Poor	33	52	33	> 300
Lake Michigan 2 (WI)	23	No	Good	Excellent	13	> 300	> 300	ND[f]
Lake Michigan 3 (WI)	23	Yes	Sufficient	Sufficient	17	31	26	36
Lake Michigan 4 (MI)	27	Yes	Excellent	Excellent	7	21	21	98.5
Lake Michigan 5 (MI)	27	Yes	Good	Excellent	7	53	53	74
Lake Michigan 6 (MI)	12	No	Excellent	Excellent	17	292	292	201
Total	287					21	54	> 300

[a]United States Environmental Protection Agency, 2012 recreational water-quality criteria (USEPA 2012). Yes = beach met both the geometric mean (GM) of 35 CFU/100 mL and statistical threshold value (STV) of 130 CFU/100 mL values for enterococci based on the reported enterococci values over the course of the study. No = beach failed both criteria.

[b]Directive 2006/7/EC of the European Parliament and of the Council of 15 February 2006 concerning the management of bathing water quality and repealing Directive 76/160/EEC. Official Journal of the European Union L 64/37, 2006. Excellent: <100 CFU/100 mL based on a 95th percentile evaluation of 16 samples over 4 years; good: <200 CFU/100 mL based on a 95th percentile evaluation of 16 samples over 4 years; sufficient: <185 CFU/100 mL based on a 90th percentile evaluation of 16 samples over 4 years; and poor: does not meet 'sufficient'.

[c]Directive 2006/7/EC of the European Parliament and of the Council of 15 February 2006 concerning the management of bathing water quality and repealing Directive 76/160/EEC. Official Journal of the European Union L 64/37, 2006. Excellent: < 200 CFU/100 mL based on a 95th percentile evaluation of 16 samples over 4 years; good: <400 CFU/100 mL based on a 95th percentile evaluation of 16 samples over 4 years; sufficient: <330 CFU/100 mL based on a 90th percentile evaluation of 16 samples over 4 years; poor: does not meet sufficient.

[d]Beach action value (BAV), United States Environmental Protection Agency, 2012 recreational water-quality criteria (USEPA 2012); 70 CFU/100 mL.

[e]NA, beach did not have any sample that exceeded BAV; therefore, no value could be determined.

[f]ND, not determined because three samples exceeded ENT BAV but staphylococci concentration was not quantifiable.

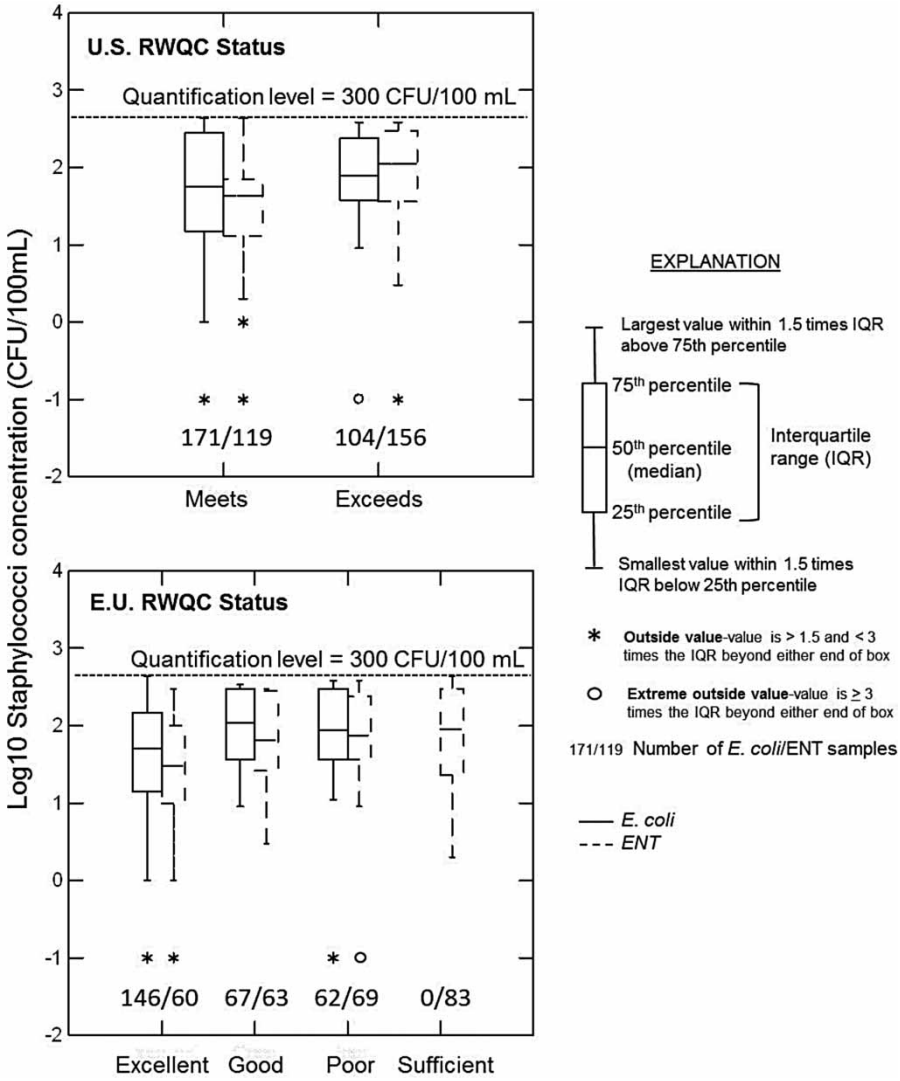

Figure 2 | Box plot of staphylococci concentrations at beaches grouped with respect to US and EU recreational water-quality criteria (RWQC) status based on *E. coli* or enterococci (ENT) concentrations.

correlation was significant at $P < 0.05$ (Spearman's rho = 0.571, −0.200, and 0.128, respectively).

The *femA* and *mecA* genes were detected in samples from beaches that exceeded as well as beaches that met water-quality criteria (Table 4). The *femA* gene was detected in 17% of samples from beaches that met the US RWQC criteria for *E. coli*, 15% of samples from beaches that would have been designated as excellent by the EU coastal water-quality criteria for *E. coli*, 11% of samples from beaches that met the US RWQC ENT criteria, and 10% of samples from beaches designated as excellent by the EU coast ENT criteria (Table 4). The Kruskal–Wallis test was used to compare frequency of gene detections between beaches that met or exceeded US RWQC criteria. The detection of *femA* was statistically significantly higher ($P = 0.011$) at beaches that exceeded the US RWQC ENT criteria compared to beaches that met the US RWQC ENT criteria. However, the *mecA* gene was more frequently detected at beaches that met US *E. coli* RWQC ($P = 0.044$). The result of a Kruskal–Wallis test followed by a Conover–Inman multiple comparison determined that 'good' beaches had a higher percentage of *mecA* gene detections than 'excellent' or 'poor' beaches. Although no statistical difference at $P < 0.05$ was found for any other gene and US or EU criteria combination

Table 3 | The percentage of samples analyzed that had a positive detection for the *Staphylococcus aureus* specific gene (*femA*) and the gene responsible for methicillin resistance (*mecA*)

Beach	Total number of samples analyzed	% of samples positive for *femA*	% of samples positive for *mecA*	% of samples positive for *mecA* and *femA*
Erie 1	23	30	26	22
Erie 2	23	35	17	13
Erie 3	23	17	30	9
Erie 4	23	43	48	30
Erie 5	17	24	18	0
Huron 1	21	0	38	0
Huron 2	21	5	38	0
Michigan 1	24	4	4	4
Michigan 2	23	22	26	17
Michigan 3	23	0	9	0
Michigan 4	27	4	44	4
Michigan 5	27	19	33	15
Michigan 6	12	42	0	0
All samples	287	18	27	9

(Table 4), *femA* + *mecA* detection frequency tended to be least ($P = 0.054$) at beaches with 'excellent' ENT water quality, and at beaches that met US ENT RWQC ($P = 0.100$).

There was no statistical difference in detections of these genes in samples that exceeded, as opposed to met, the US *E. coli* BAV (two-tailed Fisher's exact test; $P = 0.848$, 0.826, and 0.589 for *mecA*, *femA*, and *femA* + *mecA*, respectively) or the US ENT BAV ($P = 0.741$, 0.424, and 0.615 for *mecA*, *femA*, and *femA* + *mecA*, respectively).

Detection of PVL and SSC*mec* gene types

Of the 13 beaches, eight had at least one sample (27 total samples) that was positive for both *femA* + *mecA*, indicating the potential for MRSA (Table 5). The PVL gene was detected in 12 samples from six of the eight beaches with *femA* + *mecA* positive samples. All five SCC*mec* types were detected. PVL plus SCC*mec* types IV or V genes (CA-MRSA characteristic) were detected in six and four samples, respectively, and these occurred at four different study beaches.

MRSA isolation

Eight presumptive MRSA isolates (SA characteristics on BP agar and MRSA characteristics on MSA + CEF) were obtained from seven different beaches that had *femA* + *mecA* positive samples. 16S rDNA sequencing indicated >99% genetic match to *S. aureus* for all eight isolates. Only four of the eight isolates tested positive for *mecA*. The four *mecA* positive isolates were also positive for PVL. The only SCC*mec* type identified in *mecA* + PVL positive isolates was type IV.

Table 4 | Percentage of samples positive for the SA (*femA*) and methicillin resistance (*mecA*) genes based on beach-water-quality criteria for the United States (US) and European Union (EU)

	US RWQC		EU coastal water-quality status			
E. coli	**Met beach RWQC (n = 179) (%)**	**Exceeded beach RWQC (n = 108) (%)**	**Excellent (n = 156) (%)**	**Good (n = 67) (%)**	**Sufficient (n = 0)**	**Poor (n = 64) (%)**
Positive for *femA* gene	16.8	19.4	15.4	22.4	NA	18.8
Positive for *mecA* gene	**30.7**	**20.4**	**26.3**[a]	**38.8**[a]	**NA**	**15.6**[a]
Positive for *femA* + *mecA* genes	10.1	8.3	7.7	13.4	NA	9.4
Enterococci	**Met beach RWQC (*n* = 121) (%)**	**Exceeded beach RWQC (*n* = 166) (%)**	**Excellent (*n* = 60) (%)**	**Good (*n* = 73) (%)**	**Sufficient (*n* = 69) (%)**	**Poor (*n* = 85) (%)**
Positive for *femA* gene	**10.8**	**22.4**	10.0	24.7	20.3	15.3
Positive for *mecA* gene	28.3	25.5	33.3	26.0	29.0	21.2
Positive for *femA* + *mecA* genes	5.8	11.5	1.7	15.1	13.0	7.1

Bold samples were statistically different based on Kruskal–Wallis test, *P* values <0.05.
[a]Good was statistically different from 'poor' ($P = 0.003$) and 'excellent' ($P = 0.043$); 'excellent' was not statistically different from 'poor'.

Table 5 | Panton-Valentine leukocidin (PVL) gene and the staphylococcal cassette chromosome *mec* (SCC*mec*) type as determined by PCR analysis, in beach-water samples positive for both *femA* and *mecA*

Beach	Number of samples positive for *femA* and *mecA*	PVL	SCC*mec* I	SCC*mec* II	SCC*mec* III	SCC*mec* IV	SCC*mec* V	PVL + SSC*mec* IV	PVL + SSC*mec* V
Erie 1	5	5	0	1	2	3	3	3	2
Erie 2	3	2	2	2	2	2	1	1	1
Erie 3	2	1	0	0	2	1	0	1	0
Erie 4	7	2	1	3	3	4	0	0	0
Erie 5	0	NA[a]	NA	NA	NA	NA	NA	NA	NA
Huron 1	0	NA	NA	NA	NA	NA	NA	NA	NA
Huron 2	0	NA	NA	NA	NA	NA	NA	NA	NA
Michigan 1	1	0	1	0	1	0	0	NA	NA
Michigan 2	4	1	2	1	4	1	1	0	0
Michigan 3	0	NA	NA	NA	NA	NA	NA	NA	NA
Michigan 4	1	0	1	1	1	1	1	NA	NA
Michigan 5	4	1	3	3	2	2	3	1	1
Michigan 6	0	NA	NA	NA	NA	NA	NA	NA	NA
Total	27	12	10	11	17	14	9	6	4

[a]NA, there were no samples positive for both *femA* and *mecA*; therefore, subsequent PVL and SCC*mec* analyses were not done.

Intensive study at one beach location

Samples collected after noon each day had significantly greater numbers of bathers (Fisher's exact test, $P = 0.0001$), significantly higher concentrations of staphylococci (Mann–Whitney U test; $P = 0.033$), and significantly more *femA* and *femA* + *mecA* gene detections (Fisher's exact test, both $P = 0.0001$; Figure 3) than samples collected before noon.

Factors that influence staphylococci concentrations

The end-of-season GM staphylococci concentration for each beach (calculated using 300 in place of >300 CFU/100 mL) was significantly correlated ($P < 0.05$) with average percent cloud cover (Spearman's rank correlation; Spearman's rho = 0.767, $n = 12$), maximum water level difference over 24 h (0.803), percent urban land cover in the beach catchment (area draining directly to the beach; 0.727), and the percent fine-silty soils and suspended sediment yield of the two nearest watersheds (0.861 and 0.758, respectively). Using 245 daily samples having staphylococci concentrations and environmental data, log10 of the staphylococci concentration was significantly correlated with percent cloud cover on the day of sampling (0.259) and the previous day (0.301), and with wave height (standardized to the daily mean for each beach; 0.247), but not with any measure of rainfall in the 24, 48, or 72 h preceding sample collection ($P > 0.160$ for all).

Factors that influence *mecA*, *femA*, and potential MRSA detections

The percent frequency of *mecA* detections for each beach was not significantly correlated with any measured variable. Although some unmeasured variable may have influenced *mecA* detection, only two beaches had low frequencies of detection, and there may not have been sufficient variability in the data to evaluate influencing factors.

The percent frequency of the *femA* detections for each beach was significantly correlated with maximum water level difference over 24 h (Spearman's rho = 0.757, $n = 12$), population in the beach catchment (0.669), suspended sediment yield of adjacent rivers (0.640), and average percent cloud cover (0.604). Among the 273 beach-water samples with environmental data, all measures of antecedent rainfall in the catchment or watersheds were negatively correlated

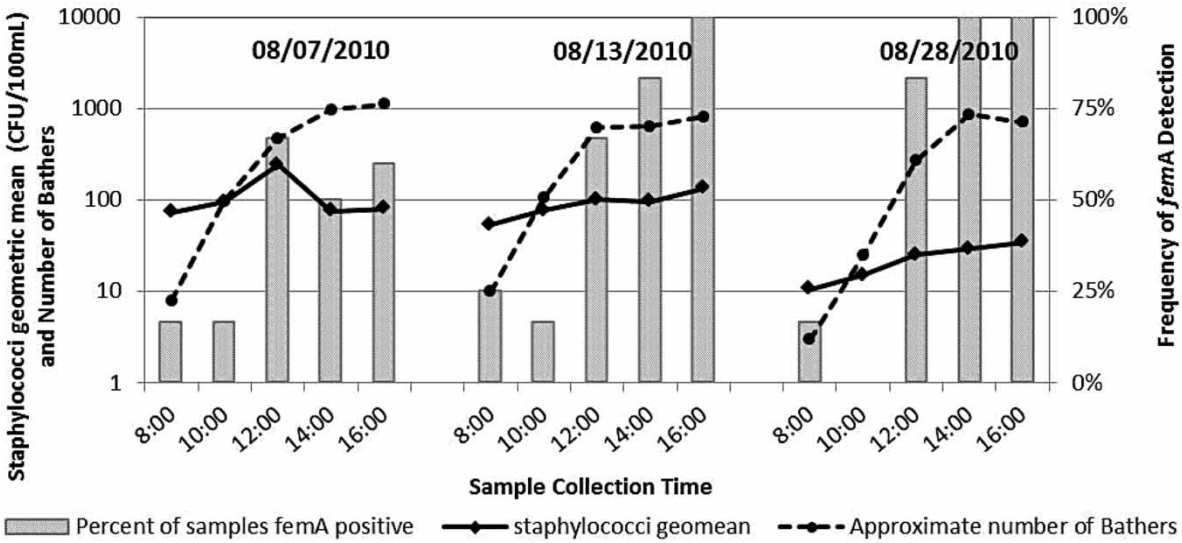

Figure 3 | Staphylococci concentrations (geomean of six locations), number of bathers, and percentage of samples that contained the *femA* gene during a single beach study that included the collection of samples every 2 h from 8:00 a.m. to 4:00 p.m. on 7, 13, and 20 August 2010.

(Spearman's rho = −0.251 to −0.027) with *femA* detection, and significantly so for rainfall measured in the beach catchment or adjacent watersheds 72 h preceding sample collection.

The detection frequency of *mecA* + *femA* at beaches was correlated with area of the beach catchment (0.660), and average beach wave height (0.57), and as for *femA* alone, potential MRSA (*mecA* + *femA*) detection was most highly correlated with beach catchment population (Spearman's rho = 0.626, $n = 12$). Also as for *femA* alone, among the 273 beach-water samples, all measures of antecedent rainfall in the catchment or watersheds were negatively correlated (Spearman's rho = −0.254 to −0.046) with *mecA* + *femA* detection, and significantly so for rainfall measured in the beach catchment or adjacent watersheds 72 h preceding sample collection.

DISCUSSION

Staphylococci concentrations were lower at beaches that met US RWQC or excellent EU criteria for *E. coli* or ENT than at beaches that exceeded US RWQC or were in other EU criteria categories. However, in this study, staphylococci concentrations were not correlated with the detection frequency of any gene. This could be a result of our methods as staphylococci concentrations were censored to 300 CFU/100 mL as a maximum value. In addition, SA and MRSA would likely constitute only a proportion of the total staphylococci in beach water. Thus, it remains unclear whether total staphylococci concentrations may be predictive of the occurrence of SA or MRSA.

The *femA* gene was detected more frequently at beaches that exceeded US ENT (but not *E. coli*) RWQC, although there was no significant relation with EU *E. coli* or ENT RWQC. There was also a tendency for *femA* + *mecA* detections to be least frequent at beaches that met US RWQC or 'excellent' EU ENT (but not *E. coli*) RWQC, although this trend was not significant at $P < 0.05$. In addition, fewer of the 13 beaches we studied met US or EU ENT RWQC, as opposed to *E. coli* RWQC. Thus, our study indicates that *E. coli* was not predictive of the presence of SA (*femA*) or MRSA (*femA* + *mecA*) at these beaches, and that a higher standard of microbiological water quality based on ENT may be needed in order to indicate the potential for SA in a water body. *E. coli* is an indicator of fecal pollution while staphylococci are found more frequently on the skin surface, and SA is considered an indicator of non-fecal shedding by swimmers for swimming pool evaluation (WHO 2006). Thus, a lack of relationship between *E. coli* and SA is reasonable. Enterococci, although often also of fecal origin, may also be native to the environment (Teixeira & Facklam 2003), and may have environmental survival

characteristics more similar to those of staphylococci. The role of ENT as an indicator of SA and MRSA should be explored further.

Results of this study suggest a process by which staphylococci, and especially SA or MRSA, are introduced to beach water from wave-washing, particularly in urban settings, or settings where soils may be conducive to entrapment and transport of bacteria, and under conditions that are protective of survival, such as increased cloud cover. SA may be rapidly inactivated in the presence of sunlight (Fujioka & Unutoa 2006), consistent with our findings of a relationship with cloud cover. Beach sand is a known reservoir for staphylococci and MRSA (Soge *et al*. 2009; Levin-Edens *et al*. 2011; Goodwin *et al*. 2012; Esiobu *et al*. 2013). Esiobu *et al*. noted that SA levels were greatest in dry sand, at beaches with high human density, and had a patchy distribution suggestive of human-release hotspots. Eichmiller *et al*. (2014) demonstrated that MRSA could grow in freshwater sand after artificial addition. Consistent with a sand influence, measures of wave-washing of beach sand, such as water level or wave height, were correlated with *femA* and potential MRSA detection frequency in our study.

Bathers are a recognized source of SA and MRSA at beaches (Charoenca & Fujioka 1993; Papadakis *et al*. 1997; Elmir *et al*. 2007; Plano *et al*. 2011; Curiel-Ayala *et al*. 2012; Goodwin *et al*. 2012). Papadakis *et al*. and Goodwin *et al*. noted a correlation between the number of swimmers and SA concentrations in beach water, and Curiel-Ayala *et al*. found the highest concentrations of SA at Mexican coastal beaches at 3 p.m., when swimmer numbers were the greatest. Similarly, our focused study at a single beach indicated a significant association between bather numbers, staphylococci concentrations, and *femA* gene detection. Our data also suggest that urban land use in the beach catchment, and catchment population, is correlated with *femA* and potential MRSA detection frequencies. This could be a result of greater beach use in urban areas.

Our findings indicate numerous SA and MRSA types, and identify the presence of CA-MRSA in ambient fresh recreational water in the US Great Lakes. MRSA (*mecA* + *femA*) was found at eight beaches and in 9% of all samples. MRSA were confirmed through the isolation and sequence identification of four SA strains from three different beaches. These isolates had both *mecA* and *femA* genes, PVL, and SCC*mec* types that have been identified in clinical CA-MRSA strains. CA- and HA-MRSA strains are genetically different (David & Daum 2010). The PVL gene, reported to be specific to CA-MRSA strains, was detected in 12 of the 27 samples that were *mecA* + *femA* positive, and 10 of the 12 samples were also positive for SCC*mec* types IV and V, which have been identified to be CA-MRSA specific. This information is supportive that CA-MRSA strains were present in these samples. Interestingly, HA-MRSA strains were also indicated based on the detection of SCC*mec* types I–III in these same samples. No isolate carried these SCC*mec* types, but the number of isolates was limited due to the scope and nature of the study. In addition to four confirmed MRSA isolates, there were four additional isolates with >99% 16S rDNA sequence similarity to SA, and that had the typical characteristics of MRSA when cultured on appropriate media, but which did not contain the *mecA* gene. While most commonly found in SA, other staphylococci species carry the *mecA* gene (Martins *et al*. 2007), and some MRSA carry an alternative *mecA* homolog (García-Álvarez *et al*. 2011). Further evaluation of these additional methicillin-resistant types was beyond the scope of this study. Other studies have reported novel MRSA strains isolated from animals and other environments with different genetic types than typical CA- or HA-MRSA types (Van Loo *et al*. 2007; Soge *et al*. 2009). To better understand the clinical significance of MRSA detected in recreational waters, it will be important to further characterize isolates with respect to typical types detected in local or regional healthcare settings (Otter & French 2010).

CONCLUSIONS

Study results indicated widespread occurrence of multiple MRSA types in fresh bathing waters. Recreational swimming is rarely considered a source of MRSA. We could find only one report suggesting a linkage between community CA-MRSA prevalence and swimming (Bothwell *et al*. 2007), and a review of marine swimming-related illness did not mention *Staphylococcus* (Henrickson *et al*. 2001). Our study contributes to the growing body of evidence, previously only available for marine waters, implicating recreational waters as a potential source for MRSA infections. As CA-MRSA infections proliferate in the USA, and increase in

prevalence in the EU (Otter & French 2010), healthcare workers should be aware of this potential source of infection.

The detection of SA or MRSA genes was related better to US or EU ENT-defined water-quality criteria than US or EU *E. coli*-defined water-quality criteria. It is important to note that if *E. coli* are the only indicator bacterium used to determine beach microbiological water quality, as is often the case in the USA, then the risk from non-fecal pathogens such as SA and MRSA will not be represented. Given the economic and healthcare burden that can be associated with infections typically caused by SA (Charoenca & Fujioka 1993; Wade *et al.* 2013) and the increased costs associated with MRSA (e.g., Rubio-Terres *et al.* 2010), monitoring of ambient recreational waters, both marine and freshwater, for non-fecal bacteria such as staphylococci and/or SA may be warranted.

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DISCLAIMER

Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the US Government.

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