Science of the Total Environment 565 (2016) 1028–1036

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Occurrence of methicillin-resistant *Staphylococcus aureus* in surface waters near industrial hog operation spray fields

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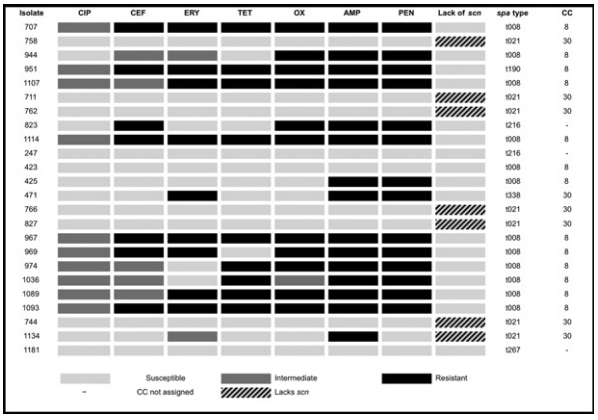
HIGHLIGHTS

- The presence of waterborne MRSA near industrial hog operations is unknown.
- We studied the presence of MRSA in surface water near industrial hog operations.
- We used a combination of culture, biochemical, and molecular confirmation methods.
- MRSA was detected in nine surface water samples.
- Both human and non-human origin *S. aureus* were present in surface water near IHOs.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 23 March 2016
Received in revised form 12 May 2016
Accepted 13 May 2016
Available online 31 May 2016

Editor: D. Barcelo

ABSTRACT

Industrial hog operations (IHOs) have been identified as a source of antibiotic-resistant *Staphylococcus aureus*, including methicillin-resistant *S. aureus* (MRSA). However, few studies have investigated the presence of antibiotic-resistant *S. aureus* in the environment near IHOs, specifically surface waters proximal to spray fields where IHO liquid lagoon waste is sprayed. Surface water samples (n = 179) were collected over the course of approximately one year from nine locations in southeastern North Carolina and analyzed for the presence of presumptive MRSA using CHROMagar MRSA media. Culture-based, biochemical, and molecular tests, as well as matrix-assisted laser desorption/ionization-time of flight mass spectrometry were used to confirm that isolates that grew on CHROMagar MRSA media were *S. aureus*. Confirmed *S. aureus* isolates were then tested for susceptibility to 16

Abbreviations: BHIB, brain-heart infusion broth; BP, Baird-Parker; IHO, industrial hog operation; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

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http://dx.doi.org/10.1016/j.scitotenv.2016.05.083
0048-9697/

Keywords:
Antibiotic resistance
Water quality
Staphylococcus aureus
MRSA
Industrial hog operations

antibiotics and screened for molecular markers of MRSA (*mec*A, *mec*C) and livestock adaptation (absence of *scn*). A total of 12 confirmed MRSA were detected in 9 distinct water samples. Nine of 12 MRSA isolates were also multidrug-resistant (MDRSA [i.e., resistant to ≥3 antibiotic classes]). All MRSA were *scn*-positive and most (11/12) belonged to a staphylococcal protein A (*spa*) type t008, which is commonly associated with humans. Additionally, 12 confirmed *S. aureus* that were methicillin-susceptible (MSSA) were recovered, 7 of which belonged to *spa* type t021 and were *scn*-negative (a marker of livestock-adaptation). This study demonstrated the presence of MSSA, MRSA, and MDRSA in surface waters adjacent to IHO lagoon waste spray fields in southeastern North Carolina. To our knowledge, this is the first report of waterborne *S. aureus* from surface waters proximal to IHOs.

1. Introduction

Since the 1980s, pork production in the United States has been characterized by a shift from small, independently owned hog operations to large, vertically integrated operations (MacDonald and McBride, 2009) often referred to as industrial hog operations (IHOs). This change in production practices has been particularly pronounced in North Carolina, which is second only to Iowa in pork production. The majority of IHOs are concentrated in the southeastern part of the state (Wing et al., 2000). One of the animal husbandry practices of IHOs is the use of antibiotics for growth promotion and disease prevention (MacDonald and McBride, 2009). Such antibiotic uses contribute to the selection of antibiotic-resistant bacteria in and around IHOs (Love et al., 2011).

A growing body of literature has documented the emergence of novel livestock-associated antibiotic-resistant *Staphylococcus aureus*, including methicillin- (MRSA) and multidrug-resistant (MDRSA) *S. aureus* strains, that colonize humans who have direct or indirect exposure to livestock. Strains of *S. aureus* with genetic and phenotypic markers of livestock adaptation have been identified in many countries, including France (Armand-Lefevre et al., 2005), the Netherlands (Huijsdens et al., 2006; Van den Broek et al., 2009), Belgium (Denis et al., 2009), Canada (Khanna et al., 2008), and the United States (Nadimpalli et al., 2014; Rinsky et al., 2013; Smith et al., 2013; Smith et al., 2009). These livestock-associated strains of *S. aureus* have also emerged in the community in areas with a high density of pig farming (Van Cleef et al., 2010).

S. aureus clonal complex (CC) 398 is a particular *S. aureus* strain commonly found in livestock and its emergence and dissemination has received global attention (Smith and Pearson, 2011). However, researchers have since documented that CC398 is not the only *S. aureus* strain circulating in the IHO environment; CC9 is being observed in hogs and IHO workers in the United States (Nadimpalli et al., 2014) and in hogs and IHO workers in Asia (Patchanee et al., 2014; Ye et al., 2016). Furthermore, among these and other lineages, there is evidence that genetic markers may distinguish livestock- from human-adapted clades among the CC398 and CC9 genotypes commonly associated with livestock (Sung et al., 2008). Price et al. (2012) demonstrated that among a group of *S. aureus* CC398 isolates, presence of *tet*(M) and absence of the *scn* gene, which encodes a staphylococcal complement inhibitor that is a part of the immune evasion cluster (IEC) in *S. aureus*, were strongly associated with *S. aureus* CC398 isolates of animal adaptation. While absence of the *scn* gene has only been validated as a marker of livestock association among certain CCs, the findings of McCarthy et al. (2011), Verkaik et al. (2011), and Sung et al. (2008) suggest that the lack of *scn* can serve as a marker of non-human origin *S. aureus* strains.

While the emergence and characterization of *S. aureus* with markers of livestock adaptation among hogs and IHO workers have become better documented and described, evidence for the dissemination of these strains to the off-farm environment is limited. Conventional IHOs in North Carolina commonly practice a waste disposal method whereby waste from hundreds to thousands of hogs is collected in open pit lagoons and then sprayed onto nearby fields as fertilizer. Land-applied IHO lagoon fecal waste can run off from spray fields into nearby creeks and streams and impair microbial water quality. This has been shown via detection of swine-specific fecal microbial source-tracking markers in surface waters proximal to IHOs in southeastern North Carolina (Heaney et al., 2015).

S. aureus and MRSA are not typically considered waterborne pathogens, but recent research has demonstrated that both clinical and environmental strains of MRSA can survive in marine and freshwaters for up to ten and five days, respectively (Levin-Edens et al., 2011a). Waterborne *S. aureus* and MRSA have been detected in recreational marine (Goodwin et al., 2012; Levin-Edens et al., 2011b; Plano et al., 2011; Plano et al., 2013; Viau et al., 2011) and fresh waters (Levin-Edens et al., 2011b). Some have suggested that the source of *S. aureus* in recreational waters is beach-goer shedding (Charoenca and Fujioka, 1993; El-Shenawy, 2005; Enns et al., 2012; Plano et al., 2011; Plano et al., 2013); however, Viau et al. (2011) found that the presence of waterborne *S. aureus* was positively associated with agricultural land covers in O′ahu, suggesting that non-human sources of waterborne *S. aureus* sources may also contribute *S. aureus* to surface waters.

Although MRSA has been recovered from industrial swine and IHO workers' noses, dust and surface samples within IHOs, and air and soil in the surrounding environment, surface waters near IHOs have yet to be evaluated for the presence of MRSA. In this study we investigated the presence of MRSA in surface water samples collected proximal to IHO lagoon waste spray fields in southeastern North Carolina—one of the densest areas of industrial hog production in the United States. We focused on presence of MRSA in surface waters proximal to IHOs because when this study was designed, the scientific literature suggested that a novel livestock-associated MRSA clade was emerging in IHOs in Europe and the United States (de Neeling et al., 2007; Huijsdens et al., 2006; Smith et al., 2009; Van den Broek et al., 2009). The goals of this study were to: 1) test surface waters proximal to IHO spray fields for the presence of MRSA; 2) investigate the phenotypic antibiotic susceptibility profiles of MRSA isolates from these surface waters; and 3) characterize MRSA isolates using genotypic methods to understand their potential origins (i.e., human- or livestock-associated).

2. Materials and methods

2.1. Study area and sample collection

Sampling locations are as described in Heaney et al. (2015); a total of nine sampling locations were included in this study (Fig. 1). The study area was located in southeastern North Carolina, a region where there is a high density of industrial hog and poultry operations (Fig. 1). Liquid waste management systems are used in the majority of IHOs in the area, whereby liquid waste is collected in large open-pit lagoons and sprayed onto nearby cropland periodically. Surface water samples were collected from public access waters proximal to swine lagoon spray fields from mid-February 2010 to mid-January 2011 as described in Heaney et al. (2015). Presumptive MRSA detection began after the first week of the study, resulting in a smaller sample size than is described in Heaney et al. (2015). Samples were transported on ice to UNC-Chapel Hill by a courier and were analyzed for presumptive MRSA within 24 h of sample collection.

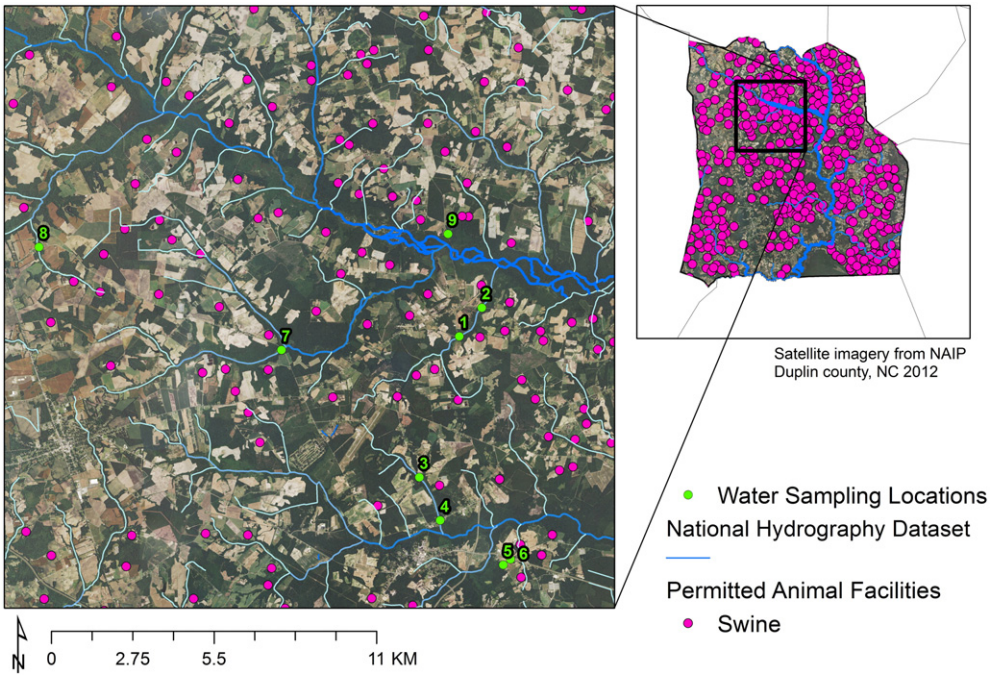

Fig. 1. Map of water sampling sites proximal to industrial hog operations and spray fields in Duplin County, NC. Adapted from Heaney et al. (2015).

2.2. Presumptive MRSA isolation

Surface water samples were analyzed for MRSA following the experimental procedure reported by Goodwin and Pobuda (2009). Water samples were filtered using a 0.45 μm membrane, placed onto CHROMagar™ MRSA (BD BBL™) plates, and incubated at 37 °C. Due to a high amount of growth on CHROMagar™ MRSA media, samples were filtered in duplicate and in multiple dilutions. Colonies with morphological characteristics of MRSA (i.e. mauve with a matte halo) were counted after 18–24 h of incubation. Up to ten of these colonies were selected from each positive sample and streaked onto CHROMagar™ Staph aureus (BD BBL™) plates for isolation and morphology verification. After incubation at 37 °C for 18–24 h, all mauve streaks with a matte halo morphology were picked and inoculated in 0.75 mL of Brain Heart Infusion Broth (BHIB) with 15% glycerol, and stored at −80 °C until further characterization.

These original archived cultures are hereafter referred to as presumptive MRSA (n = 698) because they were originally isolated on MRSA-specific culture medium. Subsequent laboratory workflow is described below and presented in Fig. 2.

2.3. Presumptive MRSA screening

In order to identify the true MRSA positives from the archived presumptive MRSA cultures (n = 698), we first performed culture-based and biochemical testing to confirm that colonies that grew on CHROMagar MRSA media were *S. aureus*. Archived presumptive MRSA isolates were regrown in 1 mL BHIB enrichment at 37 °C overnight. A loopful of inoculum was streaked for isolation on Baird-Parker (BP) agar and incubated for 48 h at 37 °C. Colonies with characteristic *S. aureus* morphology on BP agar (i.e., shiny, black colonies) at 48 h were then streaked for isolation on trypticase soy agar (TSA) and incubated

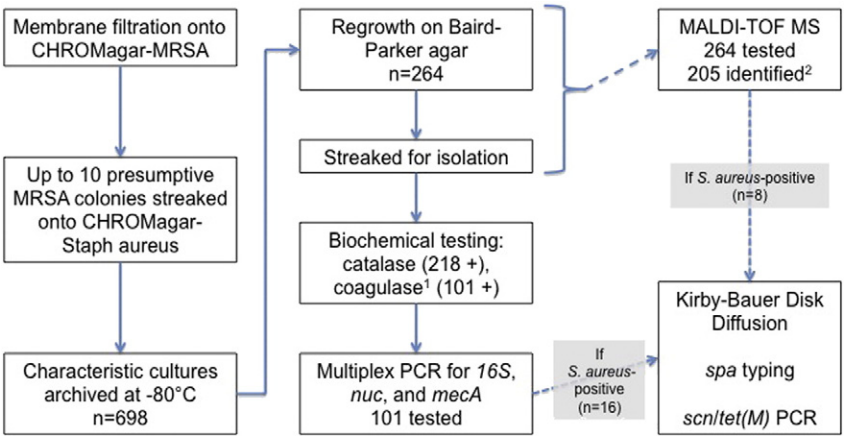

Fig. 2. Laboratory methods used to identify MRSA and MSSA from presumptive MRSA cultures. [1]Isolates were only tested for coagulase if they were catalase-positive. [2]Of the original 698 presumptive MRSA cultures. When streaking for isolation, additional sub-cultures were identified, bringing the total number of bacteria identified to at least the genus level to 227. These 227 represent 205 of the original 698 presumptive MRSA cultures.

overnight at 37 °C. Colonies were streaked again on TSA until a pure culture was obtained (n = 264). During the culture-based screening process, multiple colonies were sometimes obtained from a single original archived culture. Once a pure culture was obtained, it was first tested for the production of catalase. Catalase-positive isolates (n = 218) were then subjected to the direct tube coagulase test (BBL Coagulase Plasma, Rabbit with EDTA) according to the manufacturer's protocol. Catalase- and coagulase-positive isolates were retained as presumptive MRSA and subjected to molecular confirmation by PCR (n = 101).

2.4. Molecular confirmation of presumptive MRSA

A crude DNA extraction was performed on fresh, pure cultures (i.e., streaked for isolation on TS agar) of presumptive MRSA (n = 101) according to the protocol described in Reischl et al. (2000). All PCR reactions were then performed immediately following extraction to identify and characterize *S. aureus* isolates. A multiplex PCR was used to confirm the presence of the 16S rDNA, *nuc*, and *mec*A genes in each of the presumptive MRSA isolates with primer sequences as provided in Poulsen et al. (2003) according to the following modified PCR conditions: 2.5 μL 10× buffer (Qiagen, Valencia, CA), 5 nmol each dNTP, 5 pmol each of the 6 primers, 1 unit HotStarTaq DNA polymerase (Qiagen,Valencia, CA), and 1 μL of template. The PCR was run on a BioRad Gene Cycler ™according to the following thermal cycling parameters: initial 15 min at 95 °C; 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 60 s at 72 °C; and a final extension at 72 °C for 10 min. Presence of the *scn* and *tet*(M) genes was investigated with a duplex PCR (Stegger et al., 2013).

2.5. Matrix assisted laser desorption ionization – time-of-flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS was performed on all isolates that regrew on BP agar with characteristic *S. aureus* morphology, irrespective of the catalase, coagulase, and PCR results (n = 264) (Fig. 2). From these archived BP cultures (i.e., BHIB + 15% glycerol stocks), isolates were streaked onto TS agar and incubated overnight at 37 °C. MALDI-TOF MS was performed using the FDA-cleared VITEK MS per manufacturer's recommendations for direct colony spotting (bioMerieux, Durham, NC) (Rychert et al., 2013).

2.6. spa typing

The staphylococcal protein A (*spa*) gene was amplified using previously published PCR primers and methods (European Union Reference Laboratory for Antimicrobial Resistance, 2009). *spa* typing was performed using the Ridom Staph Type standard protocol (http://www.ridom.com) and the Ridom SpaServer (http://spa.ridom.de/index.shtml). Clonal complexes (CCs) were assigned based on existing scientific literature.

2.7. Antibiotic susceptibility profiles

Confirmed *S. aureus* isolates were tested for phenotypic susceptibility to 16 antibiotics from 11 distinct antibiotic classes. The different antibiotics and their respective concentrations are indicated in Table 1. Antibiotic susceptibility testing to all antibiotics except vancomycin was performed using the Kirby-Bauer disk diffusion method according to the protocol published by the Clinical and Laboratory Standards Institute (2012). Vancomycin susceptibility was investigated by first screening isolates on Brain-Heart Infusion agar containing 5 mg/L teicoplanin (BHIT5) (Fitzgibbon et al., 2007). Isolates were grown overnight on TS agar at 37 °C and diluted to a 0.5 McFarland standard in TS broth before streaking 10 μL on BHIT5 at 35 °C for 24 to 48 h.

Multidrug resistance was defined as resistance to three or more antibiotic classes (Magiorakos et al., 2011) and phenotypic methicillin resistance was verified by resistance to oxacillin and presence of the *mec*A gene.

Table 1
List of antibiotic concentrations used in antibiotic susceptibility testing.

Antibiotic class	Antibiotic	Concentration
Aminoglycosides	Gentamicin	10 μg
β-lactams	Ampicillin	10 μg
	Penicillin	10 units
	Oxacillin	1 μg
Cephalosporins	Ceftriaxone	30 μg
Floroquinolones	Ciprofloxacin	5 μg
	Gatifloxacin	5 μg
	Levofloxacin	5 μg
Glycopeptides	Vancomycin	Teicoplanin 5 μg/mL
Lincosamides	Clindamycin	2 μg
Macrolides	Erythromycin	15 μg
Oxazolidones	Linezolid	30 μg
Rifamycin	Rifampin	5 μg
Streptogramins	Quinupristin/dalfopristin	15 μg
Sulfanomide/methoprim	Sulfamethoxazole/trimethoprim	23.75/1.25 μg
Tetracycline	Tetracycline	30 μg

2.8. Statistical analysis

We investigated associations between MRSA positive samples and 24- and 48-hour rainfall, the swine-specific fecal microbial source tracking marker Pig-2-Bac (Heaney et al., 2015), fecal indicator bacteria (FIB [fecal coliforms, *Escherichia coli*, enterococci]) colony-forming units (CFU) per 100 mL of water, and spray field acreage within 500 and 1000 m of the sample collection site by using an analysis of covariance (ANCOVA) model, controlling for season (FIB analysis). All statistical analyses were performed in SAS 9.4 (SAS Institute Inc., Cary, NC).

3. Results

3.1. MRSA detection

Sites were sampled according to the protocol provided in Heaney et al. (2015), except samples were not tested for MRSA during the first week of the study. A total of 183 surface water samples were collected from nine sites adjacent to IHO spray fields in southeastern North Carolina. Of the 183 surface water samples tested, 179 (98%) had at least one colony that grew on CHROMagar™ MRSA (BD BBL™) with characteristic MRSA morphology, yielding 698 presumptive MRSA isolates that were archived until further characterization. We refer to these isolates as presumptive MRSA because they were originally isolated on culture medium (CHROMagar™ MRSA), which is marketed as selective for MRSA. However, this medium yielded a high proportion of false positives in our study. Of the original 698 presumptive MRSA isolates, only 12 were confirmed as MRSA. These 12 confirmed MRSA isolates were recovered from nine of the original 179 presumptive MRSA-positive surface water samples. MRSA were identified by a combination of two confirmation methods – PCR and MALDI-TOF MS. The number of isolates identified by each method is described in Section 3.5.

Fig. 3 describes the antibiotic susceptibility profiles and molecular characteristics of the 12 confirmed MRSA isolates observed in this study. Among MRSA isolates, the most common *spa* type was t008 (10/12), which belongs to clonal complex (CC) 8 (http://spa.ridom.de/mlst.shtml). Other MRSA *spa* types included t190 (CC 8) and t216 (CC unidentified). All MRSA isolates were *scn*-positive and *tet*(M) negative.

3.2. Antibiotic susceptibility profiles of MRSA

All twelve *mecA*-positive *S. aureus* (MRSA) were resistant to oxacillin. Nine of the 12 MRSA isolates exhibited multidrug resistance (i.e., were also MDRSA). Nine of the 12 MRSA isolates from eight distinct

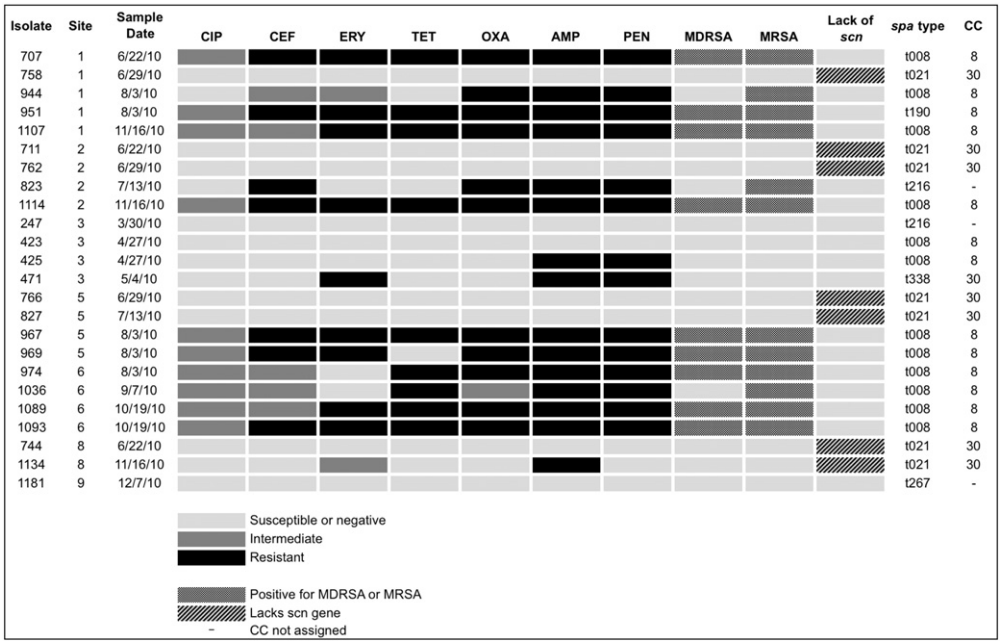

Fig. 3. Genotype and antibiotic resistance profiles of confirmed MSSA and MRSA isolates. Antibiotics include ciprofloxacin (CIP), ceftriaxone (CEF), erythromycin (ERY), tetracycline (TET), oxacillin (OXA), ampicillin (AMP), and penicillin (PEN). All isolates were susceptible to gatifloxacin, gentamycin, levofloxacin, linezolid, rifampin, quinupristin/dalfopristin, trimethoprim/sulfamethoxazole, or vancomycin. MDRSA = multidrug-resistant *S. aureus*; MRSA = methicillin-resistant *S. aureus*; CC = clonal complex.

surface water samples exhibited complete phenotypic tetracycline resistance (all were *tet*(M) negative by PCR). All of the 12 MRSA isolates were also resistant to ampicillin and penicillin. Non-susceptibility was also observed to the antibiotics erythromycin (9/12) and ciprofloxacin (10/12). No resistance to gatifloxacin, gentamycin, levofloxacin, linezolid, rifampin, quinupristin/dalfopristin, trimethroprim/sulfamethoxazole, or vancomycin was observed among MRSA isolates (Fig. 3).

3.3. MSSA detection

This study was designed to investigate the presence of MRSA in surface waters proximal to IHOs, although MSSA were also detected. Twelve isolates were identified as *S. aureus* by either PCR or MALDI-TOF MS and did not harbor *mecA* and thus were MSSA. These 12 MSSA isolates were recovered from 11 of the original 179 presumptive MRSA-positive surface water samples (2 MSSA isolates were recovered from 1 of the 11 original presumptive MRSA-positive samples). Fig. 3 describes the molecular and antibiotic susceptibility characteristics of the 12 confirmed MSSA. Seven of the 12 MSSA isolates belonged to *spa* type t021 (CC30) and were *scn*-negative. Other MSSA *spa* types included t008 (CC8; 2/12), t216 (CC unidentified; 1/12), t338 (CC30; 1/12) and t267 (CC unidentified; 1/12); MSSA belonging to these *spa* types were *scn*-positive. All MSSA were *tet*(M) negative.

3.4. Antibiotic susceptibility profiles of MSSA

Nine of 12 MSSA were susceptible to all tested antibiotics and no MSSA were multidrug-resistant. Three of 12 MSSA were resistant to ampicillin and two of 12 MSSA were resistant to penicillin. Non-susceptibility to erythromycin (2/12) was also observed. No resistance to ciprofloxacin, ceftriaxone, tetracycline, oxacillin, gatifloxacin, gentamycin, levofloxacin, linezolid, rifampin, quinupristin/dalfopristin, trimethoprim/sulfamethoxazole, or vancomycin was observed among MSSA isolates (Fig. 3).

3.5. Confirmed MRSA and MSSA identified by each detection method

The MRSA and MSSA isolates described in Sections 3.1 and 3.3, respectively, were identified by a combination of two confirmation methods – PCR and MALDI-TOF MS (Fig. 2). As our study was designed to detect MRSA from surface waters, the detection of MSSA was ancillary and an artifact of the confirmatory MRSA screening process. Additional MSSA may have been detected if a different isolation medium had been used.

A total of 16 isolates collected from 16 distinct surface water samples were confirmed as *S. aureus* by the presence of the *nuc* gene in a multiplex PCR assay, of which five harbored *mecA* and were classified as MRSA (Fig. 3). Eleven of the 16 PCR-confirmed *S. aureus* did not harbor *mec*A and were oxacillin susceptible and therefore confirmed as MSSA.

In addition, isolates were screened using MALDI-TOF MS in an attempt to identify any additional MRSA or MSSA isolates and to determine the identity of non-*aureus* staphylococci originally isolated from CHROMagar™ MRSA. As a result, eight additional *S. aureus* isolates were identified, seven of which were also *mecA* positive by PCR and therefore classified as MRSA and one of which was MSSA. The seven additional MRSA isolates identified by MALDI-TOF MS represented four distinct MRSA-positive surface water samples that would not have been captured otherwise. The additional MSSA isolate identified by MALDI-TOF MS was identified in the same surface water sample as one of the MSSA detected by PCR.

This brought the total count of confirmed waterborne *S. aureus* in this study to 24 isolates from 20 of the 179 distinct surface water samples. The 12/24 *S. aureus* that were confirmed as MRSA came from nine distinct surface water samples and the 12/24 *S. aureus* that were confirmed as MSSA isolates came from 11 distinct samples. Among isolates that originated from the same sample but were identified by different methods, no pairs of isolates were genotypically and phenotypically identical. There were no cases in which MRSA and MSSA were recovered from the same surface water sample.

3.6. Non-aureus staphylococci detected via MALDI-TOF MS analysis

Due to the high proportion of false positives detected using CHROMagar™ MRSA media, we used MALDI-TOF MS to investigate the identity of non-*S. aureus* bacteria originally isolated on what is marketed as a MRSA-specific culture medium. Isolates included in MALDI-TOF MS analysis were those that regrew on BP agar with characteristic *S. aureus* morphology, irrespective of catalase, coagulase, and PCR results (n = 264; Fig. 2). A total of 205 of the original 698 archived isolates were identified to at least the genus level. However, when streaking archived cultures for isolation, multiple colonies were sometimes obtained from a single original archived culture. Therefore, a total of 227 isolates were identified to at least the genus level, some of which represent sub-cultures of the original archived presumptive MRSA.

Staphylococcus was the most frequently detected genus (155/227). The most common non-*aureus Staphylococcus* were *Staphylococcus epidermidis* (66/227), *Staphylococcus warneri* (14/227), and *Staphylococcus saprophyticus* (11/227). Other identified *Staphylococcus* species included *Staphylococcus arlettae*, *Staphylococcus capitis*, *Staphylococcus caprae*, *Staphylococcus cohnii*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, and *Staphylococcus lugdunensis*. The next most common genera were *Bacillus* (55/227), *Enterococcus* (9/227), *Morganella* (4/227), *Acinetobacter* (1/227), *Comamonas* (1/227), *Micrococcus* (1/227), and *Prevotella* (1/227). Three species of *Bacillus*—*Bacillus cereus*, *Bacillus mycoides*, and *Bacillus thuringiensis*—were consistently indistinguishable by MALDI-TOF MS; therefore, all *Bacillus* results are reported only to the genus level. Genus- and species-level results are summarized in Table 2.

3.7. Waterborne MRSA presence and site characteristics

The association between MRSA presence versus absence and spray field acreage within 500 or 1000 m, 24- or 48-hour rainfall, FIB concentrations, and a swine-specific fecal microbial source tracking marker (Pig-2-Bac) were tested but were not statistically significant (data not shown).

Table 2
Bacterial genus and species identified by MALDI-TOF MS (n = 227).

Genus	Species	N	Percent
Acinetobacter	*Acinetobacter baumannii* complex	1	0.4
Bacillus		55	24
Comamonas	*Comamonas testosteroni*	1	0.4
Enterococcus	*Enterococcus casseliflavus*	4	1.8
	Enterococcus durans	1	0.4
	Enterococcus faecalis	2	0.9
	Enterococcus hirae	2	0.9
Micrococcus	*Micrococcus luteus/lylae*	1	0.4
Morganella	*Morganella morganii*	4	1.8
Prevotella	*Prevotella buccalis*	1	0.4
Staphylococcus	*S. arlettae*	1	0.4
	S. aureus	24	11
	S. capitis	9	4.0
	S. caprae	1	0.4
	S. cohnii ssp cohnii	2	0.9
	S. cohnii ssp cohnii/S. haemolyticus	1	0.4
	S. cohnii ssp urealyticus	5	2.2
	S. epidermidis	66	29
	S. haemolyticus	10	4.4
	S. hominis ssp hominis	9	4.0
	S. lugdunensis	2	0.9
	S. saprophyticus	11	4.8
	S. warneri	14	6.2

4. Discussion

To our knowledge, this is the first report of MRSA recovery from surface waters proximal to IHO lagoon waste spray fields, and a majority of the MRSA were also multidrug-resistant (MDRSA). During the process of confirming the presence of MRSA, we also detected MSSA. Waterborne MRSA, MSSA, and MDRSA have been reported in studies of recreational fresh and marine water beaches in the United States (Goodwin et al., 2012; Levin-Edens et al., 2011a; Levin-Edens et al., 2011b; Plano et al., 2011; Plano et al., 2013; Soge et al., 2009). All of the confirmed MRSA isolates were *scn*-positive and were assigned to *spa* types belonging to CC8 and are likely human-associated. In contrast, all MSSA isolates were *scn*-negative *spa* type t021 (CC30). Since CC30 has been identified in pigs (Agerso et al., 2012; Pomba et al., 2009) and in the nares of antibiotic-free and industrial livestock workers in North Carolina (Rinsky et al., 2013), these *scn*-negative CC30 MSSA isolates may be of non-human origin.

Some have speculated that beachgoer shedding contributes *S. aureus* to coastal recreational waters since Charoenca and Fujioka (1993) demonstrated a statistically significant association between *Staphylococcus* and bather density in marine waters in Hawaii (El-Shenawy, 2005; Plano et al., 2011; Plano et al., 2013). More recently, Levin-Edens et al. (2011b) reported that children were frequently found playing during sampling at the freshwater stream sites where MRSA was most frequently isolated. In our study, however, research team members did not observe people using any of the sample sites for recreation.

Of the original 698 presumptive MRSA isolates archived, only 24 were confirmed *S. aureus* by PCR or MALDI-TOF MS. Because our study was designed to detect MRSA, the detection of waterborne MSSA—including antibiotic-resistant MSSA—is coincidental. Therefore, it is not possible to draw any conclusions about the genotypic and phenotypic diversity of MSSA recovered in this study. One explanation for the low number of confirmed MRSA from surface waters in this study compared to previous studies may be the difference in the selective media used; other studies of waterborne *S. aureus* employed an enrichment step or media selective for *S. aureus* while we used CHROMagar™MRSA as the first line of bacterial selection for water samples. Furthermore, both Goodwin et al. (2012) and Abdelzaher et al. (2010) reported challenges associated with the use of CHROMagar™MRSA and CHROMagar™Staph aureus plates for environmental samples in which *S. aureus* is not expected to be the dominant bacterial species present in the sample. Up to 61% of samples in our study exceeded state surface water quality standards for FIB (Heaney et al., 2015), and it was common for filters on CHROMagar™ MRSA to be overgrown with non-*S. aureus* bacteria. Although Goodwin and Pobuda (2009) reported a positive predictive accuracy [sic] (positive predictive value) of 92% for CHROMagar™-MRSA using colony appearance on a filter combined with isolate appearance on agar as the test and a positive *clf*A PCR result as the reference standard, using a *Staphylococcus* enrichment method such as that described by Levin-Edens et al. (2011b) may have improved our ability to recover MRSA from surface water.

The presence of antibiotic-resistant *S. aureus* in surface waters not routinely used for recreation and in a rural agricultural setting suggests that other human and non-human sources likely contribute *S. aureus* to this environment. In rural agricultural settings, MRSA and MSSA may enter surface waters via multiple sources, including waste from human activities (Borjesson et al., 2010), wildlife (Wardyn et al., 2012), pets (Baptiste et al., 2005), or industrial animal production (Schulz et al., 2012). Approximately 49% of North Carolinians rely on private septic systems (US Census Bureau, 2011). Although MSSA and MRSA have not been evaluated in private septic system influent and effluent, MSSA and MRSA have been isolated from human sewage at wastewater treatment plants (Borjesson et al., 2010). These and other sources may have contributed MRSA and MSSA, including MDRSA, to surface waters in our study.

Our study area is influenced by a large number of IHOs. Heaney et al. (2015) detected swine-specific fecal microbial source tracking (MST) markers in the surface water sites evaluated in this study. None of the MSSA- or MRSA-positive samples were also positive for the swine-specific fecal MST markers (data not shown). Furthermore, the presence of MRSA, MSSA, or MDRSA in surface water was not associated with FIB concentrations in surface water or 24- or 48-hour rainfall (data not shown). Other ways in which *S. aureus* of potential livestock origin could enter surface water may include air (Schulz et al., 2012) and rodent or insect vectors (Ahmad et al., 2011; Graham et al., 2009; Van de Giessen et al., 2009), neither of which were examined in our research. Recent research in our study area has also documented MRSA, MSSA and MDRSA carriage in individuals who work in industrial livestock operations (Rinsky et al., 2013) and IHOs, specifically (Nadimpalli et al., 2014).

Because this study was uniquely designed to evaluate the presence of MRSA in a region dominated by IHOs, it is one of the few studies to provide information regarding waterborne *S. aureus* genotypes as well as markers of human- versus livestock-association. The most prevalent *spa* types in our study were t008 (CC8) and t021 (CC30). All seven isolates belonging to *spa* type t021 lacked *scn* and were MSSA, whereas all other confirmed MSSA and MRSA isolates were *scn*-positive. MSSA and MRSA CC30 with *spa* type t021 have been identified in pigs in Portugal (Pomba et al., 2009) and MRSA CC30 has been described in breeding pigs in Europe (Agerso et al., 2012). Additionally, *scn*-negative CC30 MSSA was recently detected in antibiotic-free and industrial livestock workers in North Carolina (Rinsky et al., 2013). Lack of *scn* in our *spa* type t021 MSSA isolates suggests that these isolates may be of non-human origin.

In contrast to the isolates with markers of livestock-association, *S. aureus* belonging to *spa* type t008 (CC8)—which is a common human-associated strain of *S. aureus*—were often methicillin- and multidrug-resistant. CC8 isolates are characteristic of USA300 strains of *S. aureus*, which is the most dominant MRSA community-associated clone in humans in the United States (DeLeo and Chambers, 2009). Thus, it is possible that *spa* type t008 MRSA, MSSA, and MDRSA observed in this study are of human origin. Multidrug-resistant *spa* type t008 MRSA was also detected in isolates from an injured Eastern cottontail rabbit (*Sylvilagus floridanus*) from a wildlife care clinic in central Iowa (Wardyn et al., 2012). In a study designed to better understand the contribution of *S. aureus* by human bathers in marine water beaches in South Florida, the sequence typing performed by Plano et al. (2013) revealed that most of their MRSA isolates were of typically human-associated genotypes, including *spa* type t008. Similarly, Soge et al. (2009) recovered sequence types commonly reported as hospital-associated at beaches in the Pacific Northwest. MSSA and MRSA *spa* type t008 have previously been recovered from both human- and non-human sources, but the *scn*-positivity of these *spa* type t008 isolates in our study suggest that they could have originated from a human source, which could include private septic systems or land application of human biosolids. The repeated detection of *scn*-positve *S. aureus spa* type t008 from surface waters in our study may suggest that this strain is able to persist longer in aquatic systems compared to livestock-associated strains of *S. aureus*. Future studies should investigate the potential differential survival of distinct *S. aureus* strains from diverse sources.

Fifteen of the twenty-four confirmed *S. aureus* isolates were resistant to at least one antibiotic. All of the antibiotics to which our collection of isolates exhibited resistance belong to antibiotic classes that have been sold or distributed for use in food-producing animals; some of which are also considered important in human medicine (Food and Drug Administration, 2011). This includes one of the *scn*-negative isolates (i.e., non-human origin), which was resistant to erythromycin and ampicillin. All of the remaining *scn*-negative isolates were susceptible to all tested antibiotics. Interpretation of these results is limited by a lack of information regarding antibiotics used in food animal production in the United States. One of the only antibiotics for which there is evidence of widespread use in food animal production, including hog production, in the United States is tetracycline (Food and Drug Administration, 2011). Tetracycline resistance was observed in nine of our twenty-four *S. aureus* isolates and all tetracycline-resistant isolates were also MRSA and MDRSA; however, all of the tetracycline-resistant isolates belong to *spa* type t008 (CC8), which is commonly associated with humans. Tetracycline is also approved for use in human medicine.

Our initial, PCR-based screening process yielded just five confirmed MRSA of the original 698 presumptive MRSA isolates, signifying a high proportion of false-positives when using CHROMagar™ MRSA media with surface water samples. MALDI-TOF MS was helpful not only to screen for additional MRSA and MSSA, but also to identify unknown bacteria that were originally isolated on media that is marketed as MRSA-selective. Results revealed that the majority (68%) belonged to the *Staphylococcus* genus, with ten non-*aureus Staphylococcus* species identified. Among other staphylococci identified, seven have been identified as *mecA* carriers (Suzuki et al., 1992). However, we did not investigate these non-aureus staphylococci for the presence of *mecA* and cannot determine the reasons for their growth on MRSA-selective media. *S. saprophyticus* has been identified in poultry flocks in Japan (Kawano et al., 1996) and pig farms in China (Wang et al., 2012). Additionally, *S. cohnii*, *S. arlettae*, *S. haemolyticus*, and *S. hominis* have previously been documented in pig farms in China (Wang et al., 2012); however, these non-*aureus Staphylococcus* species may have also been derived from a human reservoir (Kloos and Musselwhite, 1975). We are unaware of studies of these non-*aureus Staphylococcus* in swine or poultry operations in our study area.

A limitation of this study is that *S. aureus* and MRSA were not analyzed from animal or human waste, nor were samples from spray fields or industrial animal operations themselves collected. Furthermore, the density of IHOs in the region prevented us from being able to identify a referent site that was not downstream of one or more IHOs within our study area. It is possible that antibiotic-resistant *S. aureus*, including the strains we identified in this study, would be observed in surface waters that are not located proximal to IHOs. We did not observe an association between the presence of *S. aureus* and proximity to spray field acreage, rainfall, or microbial water quality data. Therefore, despite the water sampling sites' proximity to IHO spray fields, the sources of the waterborne *S. aureus* isolates in this study remain unclear. Future research should focus on better characterization of human- and non-human source samples to better understand the mechanisms by which MRSA, MSSA, and MDRSA may enter surface waters, and to better evaluate the impact of industrial animal agriculture on these bacteria in proximal surface waters.

5. Conclusions

This research demonstrated that *S. aureus*, including MRSA and MDRSA, is sometimes present in surface waters proximal to IHO lagoon waste sprayfields. Our findings are limited by our choice of a MRSA-selective media, difficulties associated with applying media adopted for clinical use to environmental samples, and our lack of a referent site or samples from IHO animals. Although the specific sources of *S. aureus* in this study are unknown, molecular analyses and genotyping revealed that both human and non-human sources may contribute *S. aureus* to surface waters in our study area. Further research appears warranted to evaluate potential mechanisms of the transport and distribution of MRSA and MDRSA into the environment.

Acknowledgments

This study would not have been possible without our strong partnership with the Rural Empowerment Association for Community Help (REACH). We would like to thank REACH organizers for their assistance with study design, sample collection and transport, and dissemination of results. The authors would also like to thank Elizabeth Christenson

for creating Fig. 1, Katie Overbey for her assistance with microbiological laboratory analysis, and Melissa Jones for her training and assistance with MALDI-TOF MS.

Funding for the water sampling component of this study was provided by the W.K. Kellogg Health Scholars Program – Community Track, Debevoise & Plimpton, LLP, and a Gillings Innovation Laboratory award from the UNC Gillings School of Global Public Health. Characterization of *Staphylococcus* was funded by NSF grant 1316318 as part of the joint NSF-NIH-USDA Ecology and Evolution of Infectious Diseases program. SMH was supported by an NSF Graduate Research Fellowship (Award No. DGE-1144081) and an NIEHS Training Grant (Award No. T32ES007018). CDH was supported by a NIOSH Award (Award No. 1K01OH010193-01A1). The funders had no role in data collection and analysis, decision to publish, or preparation of the manuscript.

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