ANTIBIOTIC RESISTANCE OF *STAPHYLOCOCCUS AUREUS* IN WATERSHEDS WITH AND WITHOUT COMMERICAL HOG OPERATIONS

Lindsay Claire Wickersham

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Approved by:

Jill Stewart

Courtney Woods

Louise Ball

ABSTRACT

Lindsay Claire Wickersham: Antibiotic Resistance of *Staphylococcus aureus* in Watersheds
With and Without Commercial Hog Operations
(Under the direction of Jill Stewart)

Antibiotic use in commercial hog operations (CHOs) can lead to selection of antibiotic resistant bacteria, which is a concern for zoonotic bacteria such as *Staphylococcus aureus*. However, the extent to which antibiotic resistant *S. aureus* from CHOs contaminates surrounding surface waters is unknown. To determine whether watersheds with CHOs have more multidrug resistant *S. aureus* (MDRSA) and livestock-associated *S. aureus* than watersheds without CHOs, *S. aureus* was isolated and characterized from surface water samples (n=44) from sites with CHOs in their watersheds (n=3) and without CHOs (n=3). *S. aureus* (n=84) was isolated from 100% of CHO sites and 66% of non-CHO sites. MDRSA (n=23) was only recovered from one CHO site on one sampling event and was positive for markers of livestock-association. No MDRSA or livestock-associated *S. aureus* was found in non-CHO sites. This research suggests that CHOs can episodically contribute livestock-associated MDRSA to surface water.

To my family and friends.
Thank you for always supporting and believing in me.

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LIST OF ABBREVIATIONS

BK   Background

CAFO   Concentrated Animal Feeding Operation

CC   Clonal Complex

CHO   Commercial hog operations

LA   Livestock-associated

MDRSA   Multidrug resistant *Staphylococcus aureus*

MPN   Most Probable Number

MRSA   Methicillin resistant *Staphylococcus aureus*

MSSA   Methicillin susceptible *Staphylococcus aureus*

pvl   Panton–Valentine leukocidin

spa   Staphylococcus Protein A

SW   Swine

CHAPTER 1: INTRODUCTION

Since 1940, there has been a marked increase in the antibiotic resistance of

Staphylococcus aureus, a zoonotic pathogen that can lead to dermatitis, respiratory and eye

infections and bacteremia (Cars, Högberg, & Murray, 2008; van Hal et al., 2012). Of particular

concern is the emergence of methicillin resistant *S. aureus* (MRSA) labeled by the CDC as a

"serious hazard level," representing an antibiotic resistant threat that will worsen without public

health intervention (CDC 2016). Infection with MRSA has been associated with increased

mortality and morbidity rates compared to infection with methicillin susceptible *S. aureus*

(MSSA)*,* and often can only be treated with intense antibiotic therapy that may cause harmful

side effects to the patient (Cosgrove et al., 2003). In addition to methicillin, *S. aureus* has

acquired resistance to several other antibiotics including those deemed of "critical importance" to

human medicine by the World Health Organization (WHO) (WHO Advisory Group on

Integrated Surveillance of Antimicrobial Resistance and World Health Organization, 2017.).

Documented cases of resistance to vancomycin in certain *S. aureus* strains emerged in 2002

(Ventola, 2015). Additionally, ceftaroline resistant strains of *S. aureus* were documented in

2011, just one year after the introduction of this critical antibiotic in 2010 (Ventola, 2015).

Despite the erratic and rapid resistance patterns emerging in *S. aureus*, a large body of research

solely investigates the emergence and presence of MRSA in various environments. Limited work

has purposefully investigated the scope of antibiotic resistance of *S. aureus* to classes of

antibiotics outside of methicillin, or even the antibiotic resistance profiles of MSSA.

Antibiotic resistant *S. aureus* is commonly documented in hospitals across the world, but

recently it has also be documented in concentrated animal feeding operations (CAFOs), particularly in commercial hog operations (CHOs) also known as industrial hog operations (IHOs) or swine CAFOs. CHOs commonly utilize antibiotics for growth promotion, with swine production alone accounting for 37% of all medically important antimicrobial drugs used in food-producing animals in the United States (FDA, 2017). Evidence of emerging antibiotic resistance not only in livestock but also in humans as a result of this practice has been well documented (Nadimpalli et al. 2016; Hatcher et al. 2016; Price et al. 2012; Gilchrist et al. 2007; Köck et al. 2013). Fecal samples from livestock and human workers on CHOs have shown intestinal flora with resistance to antibiotics without being treated clinically with the same antibiotic (Gilchrist et al., 2007). Another study investigating Dutch CHOs documented the transmission of three different MRSA strains from hogs to workers, and then from workers to their families (Köck et al., 2013).

Antibiotic resistant *S. aureus* has been isolated from livestock with increasing frequency, indicating the potential for *S. aureus* to survive in hosts outside of humans (Fitzgerald, 2012). Phenotypic and genetic differences between *S. aureus* isolated from human and livestock however provide an opportunity for identifying the source of *S. aureus* isolates (Fitzgerald, 2012). The *scn* gene for example has been found in low frequency among *S. aureus* collected from animals (2-35%) compared to humans (90-100%), thus absence of the *scn* gene can identify *S. aureus* isolates with a non-human source (Rinsky et al., 2013). Genetic sequencing and categorization of the *Staphylococcus* protein a (*spa*) gene has been used to categorize and determine clonal complexes (CCs) as a way to track populations of *S. aureus* from different hosts (Fitzgerald, 2012). This genetic categorization has identified *S. aureus* belonging to CC398, CC5, CC9, or CC30 as livestock-associated (LA) (Armand-Lefevre, Ruimy, & Andremont,

2005; Hasman et al., 2010; Hau et al., 2018; Pomba et al., 2009; Price et al., 2012; Stegger et al., 2012; Vestergaard et al., 2012). Tetracycline resistance can also be used to identify LA *S. aureus*, as tetracycline resistance is highly conserved among LA isolates (Fluit, 2012).

S. aureus and MRSA can also persist in the environment and humans exposure can occur through water systems (Charoenca and Fujioka 1995). Exposure to *S. aureus* and *MRSA* has been studied in marine water systems, encompassing potential human exposure through water, sand, and soil (Plano et al. 2013; Akanbi et al. 2017). While *S. aureus* has been studied in marine environments, little work has been done to characterize the presence and survival of *S. aureus* in freshwater. Several studies have shown that *S. aureus* can be present in fresh water surface waters in various environments, particularly those with high levels of human traffic (Fogarty et al., 2015; Levin-Edens et al., 2012; Viau et al., 2011). Yet, few studies have investigated non-human introduction of *S. aureus* into freshwater systems. Those that have addressed this topic focused more on the ability of wildlife and other animals to act as a reservoirs for *S. aureus,* rather than the surrounding environment itself (Wardyn, Kauffman, & Smith, 2012). Literature has further identified the off-site transport of microbes and antibiotic resistance from CHO lagoons and spray fields into surface waters, yet there is little research investigating the potential of CHOs to act as sources of antibiotic resistant *S. aureus* to surrounding water ways (Heaney et al., 2015; Ibekwe et al., 2002; Jokinen et al., 2012). To our knowledge, only one published study has attempted to detect MRSA in fresh water systems associated with CHOs, despite extensive evidence that *S. aureus* is present in freshwater systems, livestock, and in CHOs. Hatcher and her research team were the first to document the presence of MRSA in surface waters near CHOs; however, this research was not able to account for potential environmental sources of *S. aureus* not associated with CHOs (Hatcher et al. 2016). Additionally, this study focused on isolating

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MRSA and did not include intentional analysis of *S. aureus* isolates without resistance to methicillin.

To further expand the work of Hatcher et al (2016), this study examined surface water samples from watersheds with and without CHOs for *S. aureus*. Three background sites, with no CHOs or other known sources of fecal contamination, were included in this study to provide a baseline for presence of *S. aureus* in the environment and to serve as a comparison for potential microbial contamination. While MRSA has been widely studied in the literature, there remains limited research on MSSA and their subsequent resistance profiles to other classes of antibiotics. Here, we investigated potential emerging antibiotic resistance of *S. aureus* to a wide variety of antibiotic classes through antibiotic resistance testing of 11 different classes and intentional isolation of *S. aureus* instead of strict screening for MRSA. Furthermore, we investigated three different markers of livestock-association (LA) to provide insights into potential contamination sources: absence of the *scn* gene, tetracycline resistance, and identification of LA *spa* types.

Our objectives were to 1) determine the presence of *S. aureus* in surface waters with and without commercial hog operations in their watersheds, 2) analyze *S. aureus* collected for antibiotic resistance to 11 different mechanistic classes and 3) identify prevalence of three different livestock-associated markers among *S. aureus* isolates. This work provides an exploratory report of antibiotic resistant *S. aureus* present in surface waters proximal to CHOs in Eastern North Carolina.

CHAPTER 2: METHODS

2.1 Site Selection

Sampling locations were chosen for this study based on a USGS report by Harden (2015). A subset of three background sites (prefix: "BK") and three commercial hog operation (CHO) sites (prefix: "SW") were chosen for a total of six sampling locations. Background sites are defined as those without CHOs nor any other type of concentrated animal feeding operations (CAFO) in their watersheds. CHO sites are defined as sites with CHOs in their watershed but no other concentrated animal feeding operations (CAFOs). All watersheds sampled did not have any other known point sources of fecal waste, such as wastewater treatment plants.

The sites selected from Harden's study were the following: BK03, BK12, BK14, SW04, SW07, and SW11 (Harden, 2015). These sites were considered representative of their respective watersheds and vary in physical characteristics such as area and wetland percentage (Figure 1, Table 1). CHO sites further differed in the number of hog lagoons used for waste storage and spray field acreage, in which hog waste is sprayed onto fields as a soil conditioner (Figure 1, Table 1).

Water samples were collected from all sites seven times between December 2016 and October 2017 and one additional time in November 2016 (n=8) (Table 2). To explore the seasonal effects of *S. aureus* presence, water samples were collected twice per season for all sites, except for sites SW04, BK03, BK12, and BK14, for which only one Fall water sample was collected (Table 2). Seasons were defined according to astronomical definitions for the Northern Hemisphere.

Table 1. Sampling Site Watershed Characteristics

Site ID	Watershed area (mi^2)	Wetland percentage of watershed area	Spray field acreage in watershed	n lagoons in watershed
BK03	3.67	14%	0	0
BK12	3.55	14%	0	0
BK14	13.27	27%	0	0
SW04	1.23	15%	98.4	1
SW07	1.25	18%	46.2	1
SW11	1.95	22%	324	2
Total	24.91	109%	468	4

 Note: Background sites (prefix "BK") refer to sites without a commercial hog operation (CHO) or other known point source of fecal contamination in their watersheds. CHO sites (prefix "SW") refer to sites with a CHO in their watersheds but no other concentrated animal feeding operations (CAFOs) or other known point sources of fecal contamination. Data courtesy of Elizabeth Christenson.

Table 2. Sampling Dates and Presence of *S. aureus*

Site ID	Sampling Dates							
	11/28/16*	1/16/17*	2/20/17	4/03/17	5/09/17	6/26/17	8/14/17	10/5/17
BK03		Sample	Sample	Sample	*S. aureus* present	*S. aureus* present	Sample	Sample
BK12		Sample	Sample	Sample	Sample	Sample	Sample	Sample
BK14		Sample	Sample	Sample	Sample	*S. aureus* present	Sample	Sample
SW04		Sample	Sample	Sample	*S. aureus* present	Sample	Sample	*S. aureus* present
SW07	*S. aureus* present	*S. aureus* present	Sample	Sample	Sample	Sample	Sample	Sample
SW11	*S. aureus* present	Sample	Sample	Sample	*S. aureus* present	Sample	*S. aureus* present	*S. aureus* present
Season	Fall	Winter	Winter	Spring	Spring	Summer	Summer	Fall

☐ Water Sample Collected   ‖‖‖ *S. aureus* present

*Most probable number (MPN) calculations not performed
Note: Background sites (prefix "BK") refer to sites without a commercial hog operation (CHO) or other known point source of fecal contamination in their watersheds. CHO sites (prefix "SW") refer to sites with a CHO in their watersheds but no other concentrated animal feeding operations (CAFOs) or other known point sources of fecal contamination. Seasons are defined in accordance with the astronomical definitions for the Northern Hemisphere.

Background Sites

A. BK03

Commerical Hog Operation (CHO) Sites

D. SW04

B. BK12

E. SW07

C. BK14

F. SW11

Legend

■ Sampling Locations
▢ Watershed
═ state roads
● Swine CAFO centroid
▨ Sprayfield
▨ Cropland Data Layer Wetland
▨ 100m river buffer

River: stream order
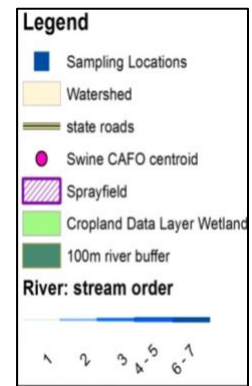
1 2 3 4-5 6-7
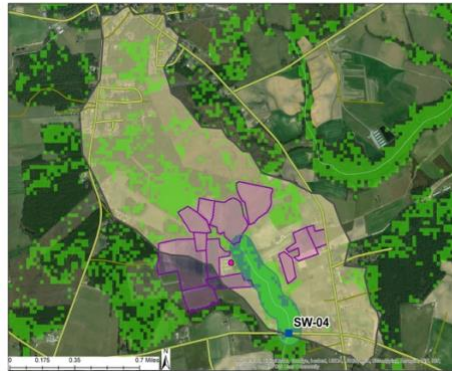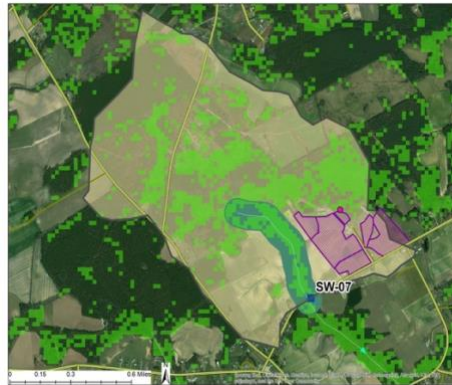
Figure 1. Maps of Sampling Sites and Watershed Information

Visual representations of sampling site locations and watershed information. Figures A-C represent background sites while Figures D-F represent CHO sites. All sites shown have no other known sources of fecal contamination other than those documented. Maps created by Elizabeth Christenson and used with permission.

A multi-meter measured ambient air temperature, water temperature, pH, conductivity, and dissolved oxygen (DO) (mg/L) at each site. One liter of water was collected at the sampling site from the center of the water bodies using sterilized containers and standard sampling technique. Water samples were placed on ice for the duration of sampling, and refrigerated at 4°C overnight. Samples were processed within 24 hours.

2.2 Processing of Water Samples and *S. aureus* Isolation

Water samples were processed using standard membrane filtration technique with 0.45 µm pore size filters (Millipore Sigma, Burlington, Massachusetts). Five volumes of 10 ml and five volumes of 50 ml samples of water were processed from each site for a total of 10 membranes per sampling site. To enrich the samples, membranes were folded into quarters using sterile tweezers and submerged in 10 ml of Mueller Hinton broth + 6.5% NaCl contained in a test tube. Tubes containing broth and membranes were vortexed at medium speed and incubated at 37° C for 24 hours. Tubes were removed from the incubator and vortexed for 3-5 seconds on medium speed before returning to the incubator for an additional 24 hours. After a total of 48 hours each tube was vortexed and broth from each tube was streaked to isolation on its own 100 mm CHROMagar™ Staph Aureus plate (Chromagar, Springfield, NJ). Biofilms and the membrane were avoided when transferring the broth to limit the presence of mixed colonies on plates. Plates were incubated at 37° C for 18-24 hours.

In accordance with the manufacturer's instructions, pink to mauve colonies with morphologies indicative of *S. aureus* were streaked to isolation on 100 mm CHROMagar™ Staph Aureus plates (Chromagar, Springfield, NJ). This process was repeated with up to five colonies from each plate. Plates were incubated at 37° C for 18-24 hours. Isolated colonies were transferred to quartered 100 mm BBL™ Mannitol Salt Agar (BD, Sparks, MD) plates and

incubated at 37° C for 18-24 hours. Isolates positive for mannitol fermentation as indicated by yellow growth were considered presumptive *S. aureus* and transferred to 1 ml of Brain Heart Diffusion Broth with 15% glycerol, vortexed, and frozen at -80° C for further analysis.

Most probable number (MPN) calculations were performed based on the number of enrichment tubes that were positive for presence of confirmed *S. aureus* identified by the confirmation testing described below. Calculations were performed in MATLAB™ and normalized to MPN per 100 ml. The mean seasonal MPNs from CHO and background sites were compared using a paired t-test in Microsoft Excel to determine significance (α=0.05).

2.3 Confirmation Testing of *S. aureus*

Biochemical and molecular assays were used to confirm *S. aureus*. All presumptive isolates were measured for production of catalase using standard methods and subjected to direct tube coagulase testing using BBL™ Coagulase Plasma Rabbit with EDTA in accordance with the manufacturer's protocol (BD, Sparks, MD). Isolates positive for both assays were subjected to molecular confirmation.

Molecular confirmation was performed on crude DNA extracts from freshly streaked isolates on TSB agar, as described by Reishcl et al. (2000). A multiplex PCR reaction and gel electrophoresis was then used to identify the presence of the following 5 genes: *Staphylococcus aureus* protein A (*spa*), *mecA*, *mecC, scn,* and *pvl* as described by Steggar et al. (2012) and as adapted by Hatcher et al. (2017). All presumptive isolates positive for the presence of the *spa* gene were considered confirmed *S. aureus* isolates and are henceforth designated simply as isolates. Two different methicillin resistance genes, *mecA* and *mecC* were tested to determine the presence of methicillin resistant *S. aureus* (MRSA) (Hatcher et al. 2017). Presence of two virulence genes was also assessed using PCR including *scn* which is a human-associated immune

evasion cluster and the Panton–Valentine leukocidin (*pvl)* gene associated with the dissemination of community-acquired MRSA (Fluit, 2012; Van Wamel, Rooijakkers, Ruyken, Van Kessel, & Van Strijp, 2006).

2.4 Antibiotic Susceptibility Testing of *S. aureus*

All confirmed *S. aureus* isolates were measured for phenotypic susceptibility to 15 different antibiotics from 11 classes (Table 3). Antibiotic susceptibility testing was performed using the Kirby Bauer Disk Diffusion method in accordance with the standardized protocol by the Clinical and Laboratory Standards Institute (CLSI 2015). All zones of inhibition except for spectinomycin and linocomyin were interpreted in accordance with the Clinical and Laboratory Standards Institute (CLSI 2015). Resistance to spectinomycin and lincomyocin was defined as having no zone of inhibition around antibiotic disks and intermediate resistance was not measured in this study because CLSI standards are not available for determination of *S. aureus* susceptibility for these two antibiotics. In accordance with Magiorakos et al. (2012), isolates that exhibited resistance to ≥ 1 antibiotic class were classified as antibiotic resistant *S. aureus*, while those that exhibited resistance to ≥ 3 antibiotic classes were classified as multidrug resistant *S. aureus* (MDRSA).

2.5 Livestock-Associated Markers

Three different livestock-associated (LA) markers were assessed to determine the potential sources of *S. aureus* to surface waters: tetracycline resistance, absence of *scn*, and *spa* type. Isolates that showed resistance to tetracycline were considered positive for one LA marker (Nadimpalli et al. 2016; Gilchrist et al. 2007; Hatcher et al. 2017). Isolates that tested negative for *scn* by PCR were considered to be associated with animals, in accordance with Nadimpalli et al. (2016). Thus isolates with an absence of *scn* were considered positive for a putative LA

marker. *Spa*-typing of isolates was conducted through Sanger sequencing of PCR products by Eton Biosciences, Inc. (Research Triangle Park, NC) in accordance with Hatcher et al. (2017). Sequences were then characterized using the Ridom StaphType software and the Ridom SpaServer (http://spa.ridom.de/index.shtml), and assigned to clonal complexes (CC) based on existing scientific literature. *Spa* types belonging to CC9, CC5, CC30, and CC398 were classified as LA *spa* types as specified in the literature and considered positive for a putative LA marker (Skallerup et al. 2015; Khanna et al. 2007.).

Table 3. Characteristics of Antibiotics Used in Antibiotic Susceptibility Testing

Antibiotic Class	Name (Abbreviation)	Disc Potency	Medical Use
Aminoglycosides	Gentamicin (GM)	10 µg	V/H (39)
	Spectinomycin (SPT)	100 µg	V
Cephalosporins	Cefoxitin (FOX)	30 µg	H
Fluoroquinolones	Ciprofloxacin (CIP)	5 µg	H
	Levofloxacin (LVX)	5 µg	H
Lincosamide	Clindamycin (CC)	2 µg	H
	Lincomycin (L)	2 µg	V
Macrolides	Erythromycin (E)	15 µg	V/H (3)
Oxazolidinone	Linezolid (LZD)	30 µg	H
Penicillians	Amoxicillin/ Clavulanic acid (AmC)	20/10 µg	V/H (0.6)
	Penicillin (P)	10 U	V/H (0.6)
Rifamycins	Rifampin (RA)	5 µg	H
Streptogramins	Quinupristin/Dalfopristin (SYN)	15 µg	H
Sulfonamides	Sulfamethoxazole/Trimethroprim (SXT)	23.75/1.25 µg	H
Tetracylines	Tetracycline (TE)	30 µg	V/H (57)

Note: V=Veterinary usage, H=Human usage, V/H(#) Veterinary and human usage. The number represents the corresponding percentage used in veterinary medicine compared to human medicine. Data courtesy of Personal Communication with Sarah Rhodes and collected from FDA (2017).

CHAPTER 3: RESULTS

3.1 Presence of *S. aureus* by Sampling Site

To determine the extent of surface water contamination of drug resistant *S. aureus* as related to CHOs in Eastern North Carolina, water samples were collected from three background sites (prefix: BK) and three commercial hog operation (CHO) sites (prefix: SW) and analyzed for presence of *Staphylococcus aureus*. *S. aureus* was recovered in 67% of background sites and in 100% of commercial hog operation (CHO) sites. *S. aureus* was present in 11 out of the total 44 water samples (25%) at all time points: present in eight samples taken from CHO sites (18%) and three samples in background sites (6.8%) (Table 2, Table 4). Antibiotic resistant *S. aureus* *(*resistant to $\geq$ 1 antibiotic class) was present in samples twice (4.5%) and multidrug resistant *S. aureus* (MDRSA*;* resistant to $\geq$ three different antibiotic classes) was present once (2.3%) (Table 4). No antibiotic resistant *S. aureus* or MDRSA was present in samples collected from background sites (Table 4). *S. aureus* with LA marker absence of *scn* was present in eight samples (18%): three times in background sites (14%) and five times in CHO sites (22%). *S. aureus* with LA marker tetracycline resistance was present in samples once (2.3%) and *S. aureus* with LA *spa* types was present in samples once (2.3%) (Table 4). No *S. aureus* with LA tetracycline resistance or LA *spa* types were present in background sites (Table 4). *S. aureus* positive for all three LA markers was present once in a CHO site and not present in background sites (Table 4). No *S. aureus* was cultured from background site BK12 during the study (Table 4).

***Table 4***: Presence of *Staphylococcus aureus* by Sampling Site

Sampling Site ID	Number of Samples	*S. aureus*	Antibiotic Resistant *S. aureus*	MDRSA	LA absent *scn*	LA tetracycline resistance	LA *spa* Type	All Three LA Markers
BK03	7	2 (29%)	0 (0%)	0 (0%)	2 (29%)	0 (0%)	0 (0%)	0 (0%)
BK12	7	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
BK14	7	1 (14%)	0 (0%)	0 (0%)	1 (14%)	0 (0%)	0 (0%)	0 (0%)
SW04	7	2 (29%)	1 (14%)	1 (14%)	2 (29%)	1 (14%)	1 (14%)	1 (2.3%)
SW07	8	2 (25%)	1 (13%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
SW11	8	4 (50%)	0 (0%)	0 (0%)	3 (38%)	0 (0%)	0 (0%)	0 (0%)
Total	44	11 (25%)	2 (4.5 %)	1 (2.3%)	8 (18%)	1 (2.3%)	1 (2.3%)	1 (2.3%)

Note: Background sites (prefix "BK") refer to sites without a commercial hog operation (CHO) in their watersheds. CHO sites (prefix "SW") refer to sites with a CHO in their watersheds but no other concentrated animal feeding operations (CAFOs) or other known point sources of fecal contamination. Antibiotic resistant *S. aureus* is defined as resistant to ≥ 1 antibiotic class. Multidrug resistant *S. aureus* (MDRSA) is defined as isolates resistant to ≥ 3 different antibiotic classes. LA is abbreviated for livestock-associated.

3.2 Most Probable Number Calculations

Most probable number (MPN) calculations were used to quantify the presence of *S. aureus* in sampling sites seasonally. The minimum MPN was <0.168 colony forming units (CFU) per 100 mL and represented zero positive enrichment tubes (Figure 2). Below detect values were denoted as 0.168 in Figure 2. The maximum MPN measured by this study was 3.76 CFU/100 mL and was measured at a CHO site in the spring (Figure 2). CHO sites had higher 3rd quartiles and maximum CFUs than background sites for all seasons except winter (Figure 2). CHO sites had a higher median CFU (0.490, 0.588) in the spring and fall compared to background sites (<0.168, <0.168) (Figure 2). No significant difference was found between mean MPNs for background and swine sites during any season (α=0.05; data not shown). Only one sample from each season were used in the MPN calculation for Fall and Winter. No enrichment tubes used in the MPN calculation were positive for *S. aureus* in the winter (Table 2, Figure 2).

Most Probable Number (MPN) of *S. aureus* in Surface Water Samples
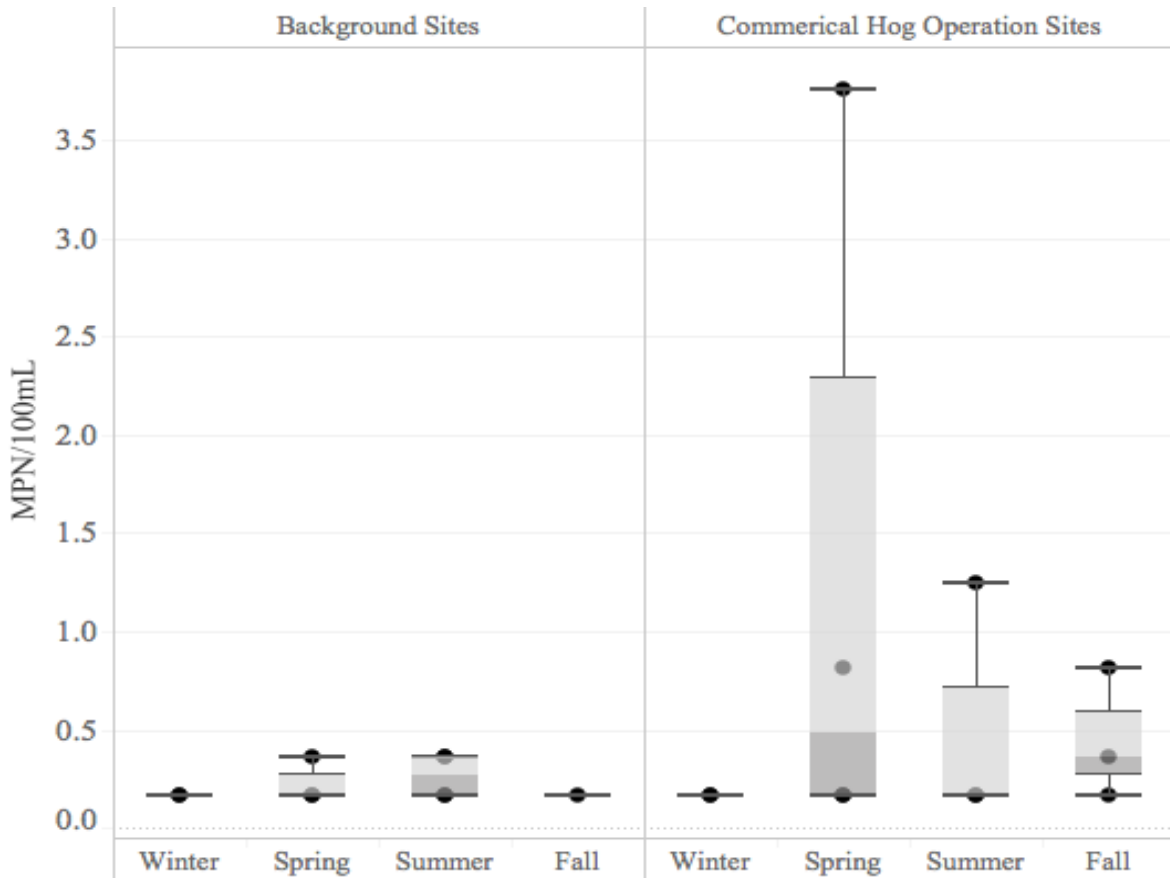
Site type	Statistics (MPN/100 ml)	Winter	Spring	Summer	Fall
	Maximum	0.168	0.365	0.365	0.168
	3rd Quartile	0.168	0.266	0.365	0.168
Background	Mean	0.168	0.234	0.299	0.168
	Median	0.168	0.168	0.266	0.168
	1st Quartile	0.168	0.168	0.168	0.168
	Minimum	0.168	0.168	0.168	0.168
	Maximum	0.168	3.76	1.25	0.811
	3rd Quartile	0.168	2.28	0.710	0.588
Commercial Hog Operation	Mean	0.168	0.873	0.349	0.448
	Median	0.168	0.490	0.168	0.365
	1st Quartile	0.168	0.168	0.168	0.266
	Minimum	0.168	0.168	0.168	0.168

Figure 2. Most Probable Number (MPN) of *S. aureus* in Surface Water Samples

Note: Most probable number (MPN) statistics of *S.* aureus per 100 ml of water samples as differentiated by type of sampling site and season. Seasons were defined according to astronomical definitions for the Northern Hemisphere. Only one sampling date was used to calculate the MPN of Fall and Winter, compared to two in Spring and Summer.

3.3 Isolate Analysis from Positive Samples

Originally 90 presumptive *S. aureus* isolates were collected, with up to five isolates being collected per sample (Table 5). This number was reduced to 85 based on results of biochemical and molecular confirmatory tests and finalized at 84 isolates with the removal of one isolate due to laboratory error (Table 5). These 84 isolates were subjected to antibiotic testing for 15 antibiotics comprising 11 different mechanistic classes. 70 (82%) of these isolates originated from CHO sites and 14 (17%) were obtained from background sites (Table 5). 27 (39%) of isolates from CHO sites exhibited antibiotic resistance to 1 or more antibiotic classes (Table 5). No antibiotic resistant isolates were obtained from background sites (Table 5). 23 (33%) isolates from CHO sites were classified as MDRSA and were positive for LA tetracycline resistance, and eight (11%) were positive for LA *spa* types (Table 5). Eight isolates (11%) from CHO sites were positive for all three LA markers (Table 5). No isolates from background sites were classified as MDRSA, presented with LA tetracycline resistance, LA *spa* types, nor presented with all three LA markers (Table 5). 61 (73%) isolates had an absent *scn*: 14 (100%) originating from background sites and 47 (67%) originating from CHO sites. No confirmed *S. aureus* isolates were identified by this study that harbored the *mecA, mecC, or pvl* gene.

Table 5: Characteristics of *Staphylococcus aureus* Isolates

Sampling Site ID	# of Presumptive *S. aureus* isolates (% of Combined Total)	# of Confirmed *S. aureus* isolates (% of Combined Total)	Antibiotic Resistant *S. aureus*	MDRSA	LA absent *scn*	LA tetracycline resistance	LA *spa* Type	All Three LA Markers
BK03	13	9	0	0	9	0	0	0
BK12	0	0	0	0	0	0	0	0
BK14	6	5	0	0	5	0	0	0
BK Total	19 (21%)	14 (17%)	0 (0%)	0 (0%)	14 (100%)	0 (0%)	0 (0%)	0 (0%)
SW04	30	30	23	23	30	23	8	8
SW07	9	9	4	0	0	0	0	0
SW11	32	31	0	0	17	0	0	0
SW Total	71 (78%)	70 (82%)	27 (39%)	23 (33%)	47 (67%)	23 (33%)	8 (11%)	8 (11%)
Combined Total	90 (100%)	84 (100%)	27 (32%)	23 (27%)	61 (73%)	23 (27%)	8 (9.5%)	8 (9.5%)

(%)=Percentage of confirmed isolates for specified site type (unless otherwise specified)

Note: Background sites (prefix "BK") refer to sites without a commercial hog operation (CHO) in their watersheds. CHO sites (prefix "SW") refer to sites with a CHO in their watersheds but no other concentrated animal feeding operations (CAFOs) or other known point sources of fecal contamination. Antibiotic resistant *S. aureus* is defined as resistant to ≥ 1 antibiotic class. Multidrug resistant *S. aureus* (MDRSA) is defined as isolates resistant to ≥ 3 different antibiotic classes. LA is abbreviated for livestock-associated.

3.4 Unique Isolate Characterization and Analysis

Due to the potential for cloning with enrichment methods, isolates were further characterized as "unique" if they harbored an original *spa* type and/or antibiotic resistance profile for their enrichment tube. By this definition, 24 unique isolates were identified with 20 (77%) obtained from CHO sites and four (17%) obtained from background sites (Table 6). Eight unique isolates (40%) obtained from the CHO sites were characterized as MDRSA and positive for LA tetracycline resistance (Table 6). Four unique isolates (20%) obtained from CHO sites were positive for all three LA markers (Table 6). No MDRSA, tetracycline resistant, nor *S.*

aureus with a LA *spa* type were isolated from background sites (Table 6). All 14 unique isolates

from background sites were absent *scn*, compared to 15 (75%) of isolates from CHO sites (Table

6). No isolates from background sites presented with all three LA markers (Table 6).

Table 6. Unique *Staphylococcus aureus* Isolates Counts and Percent Positive For Traits of
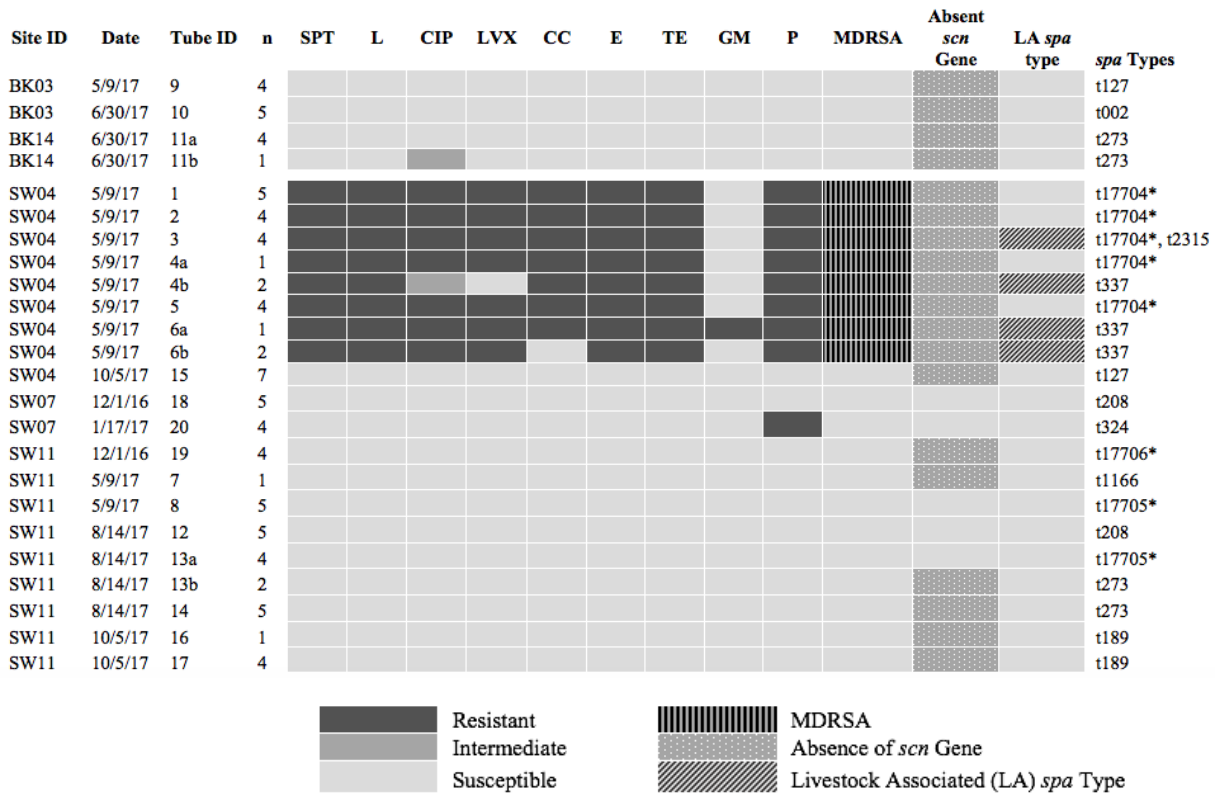Interest

Source	*n*	Antibiotic Resistant *S. aureus*	MDRSA	LA absent *scn*	LA tetracycline resistance	LA *spa* Type	All Three LA Markers
Background Site (n=3)	4 (17%)	0 (0 %)	0 (0 %)	4 (100 %)	0 (0 %)	0 (0 %)	0 (0%)
CHO Site (n=3)	20 (83%)	9 (45%)	8 (40%)	15 (75%)	8 (40%)	4 (20%)	4 (20 %)
Total	24 (100%)	9 (38%)	8 (35%)	19 (79%)	15 (63%)	4 (17%)	4 (17 %)

Note: Background sites indicate sampling locations without a commercial hog operation (CHO) in their watersheds. CHO sites indicate sampling locations with a CHO in their watersheds but no other concentrated animal feeding operations (CAFOs) or other known point sources of fecal contamination. Antibiotic resistant *S. aureus* is defined as resistant to ≥ 1 antibiotic class. Multidrug resistant *S. aureus* (MDRSA) is defined as isolates resistant to ≥ 3 different antibiotic classes. LA is abbreviated for livestock-associated.

Antibiotic resistance profiles of these 24 unique isolates were compared and analyzed.

Resistance to the following nine antibiotics was identified: spectinomycin (SPT) (n=8),

lincomycin (L) (n=8), ciprofloxacin (CIP) (n=7), levofloxacin (LVX) (n=7), clindamycin (CC)

(n=7), erythromycin (E) (n=8), tetracycline (TE) (n=8), gentamicin (GM) (n=1) , and penicillin

(P) (n=9) (Figure 3). All isolates were susceptible to the following six antibiotics: rifampin (RA),

quinupristin-dalfopristin (SYN), sulfamethoxazol-trimethoprim (SXT), linezolid (LZD),

cefoxitin (FOX), and amoxicillin-clavulanate acid(AMC) (Figure 3). All eight unique MDRSA

unique isolates were found on 5/9/17 from one sampling site, SW04. Outside of these eight

unique isolates, only two (7.7%) showed resistance (P, n=1) or intermediate susceptibility (CIP,

n=1) to antibiotics (Figure 3). All other isolates (n=15, 56%) showed susceptibility to all

antibiotics measured (Figure 3).

Sub-typing of isolates was performed through Sanger genetic sequencing of the staphylococcal protein A (*spa*). 12 *spa* types were identified using the Ridom SpaServer database (http://spa.ridom.de/index.shtml): t002 (n=1), t127 (n=2), , t189 (n=2), t208 (n=2), t273 (n=4), t324 (n=1), t337 (n=3), t1166 (n=1), t2315 (n=1), t17704 (n=5), t17705 (n=2), and t17706 (n=1) (Figure 3). Livestock-associated *spa* types (t337, t2315) were identified in the unique isolates four (17%) times (Figure 3). The following three *spa* types were identified for the first time in this study and added to the Ridom SpaServer: t17704, t17705, and t17706. Livestock-associated spa-types were also only found at one sampling time and location, 5/9/17 at SW04.

This study recovered *S. aureus* (n=84) from surface water samples from 100% of CHO sites (n=3) and 67% of BK sites (n=3). 24 unique isolates were identified from the original 84 isolates and differentiated by their unique resistance profiles and/or *spa* type within their enrichment tubes. Regardless of grouping, MDRSA and *S. aureus* isolates with all three markers of LA were found only on 05/9/17 from site SW04. All isolates with a LA *spa* type were MDRSA. The only other antibiotic resistance observed was to penicillin in a CHO isolate and intermediate ciprofloxacin resistance in one BK isolate. No MDRSA, antibiotic resistant *S. aureus, S. aureus* with a LA *spa* type, or LA tetracycline resistant *S. aureus* was isolated from background sites. All isolates from background sites were absent *scn*, a LA marker indicating a non-human source.

Site ID	Date	Tube ID	n	SPT	L	CIP	LVX	CC	E	TE	GM	P	MDRSA	Absent scn Gene	LA spa type	spa Types
BK03	5/9/17	9	4													t127
BK03	6/30/17	10	5													t002
BK14	6/30/17	11a	4													t273
BK14	6/30/17	11b	1													t273
SW04	5/9/17	1	5													t17704*
SW04	5/9/17	2	4													t17704*
SW04	5/9/17	3	4													t17704*, t2315
SW04	5/9/17	4a	1													t17704*
SW04	5/9/17	4b	2													t337
SW04	5/9/17	5	4													t17704*
SW04	5/9/17	6a	1													t337
SW04	5/9/17	6b	2													t337
SW04	10/5/17	15	7													t127
SW07	12/1/16	18	5													t208
SW07	1/17/17	20	4													t324
SW11	12/1/16	19	4													t17706*
SW11	5/9/17	7	1													t1166
SW11	5/9/17	8	5													t17705*
SW11	8/14/17	12	5													t208
SW11	8/14/17	13a	4													t17705*
SW11	8/14/17	13b	2													t273
SW11	8/14/17	14	5													t273
SW11	10/5/17	16	1													t189
SW11	10/5/17	17	4													t189

Legend: Resistant, Intermediate, Susceptible; MDRSA, Absence of *scn* Gene, Livestock Associated (LA) *spa* Type

*= Newly Identified *spa* type

Figure 3. Antibiotic Resistance Profiles and Livestock-Association of Unique *S. aureus* Isolates

Note: Isolates displayed antibiotic resistance to the following: spectomycin (SPT), lincomycin (L), ciprofloxacin (CIP), levofloxacin (LVX), clindamycin (CC), erythromycin (E), tetracycline (TE), gentamicin (GM), and penicillin (P). All isolates were susceptible to the following antibiotics: rifampin (RA), quinupristin (SYN), sulfamethoxazole (SXT), linezolid (LZD), cefoxitin (FOX), amoxicillin (AMC). LA is abbreviated for livestock-associated. To our knowledge, *spa* types t17704, t17705, t17706 were identified for the first time in this study.

CHAPTER 4: DISCUSSION

4.1. *S. aureus* presence and seasonality

This is the first exploratory study to evaluate *Staphylococcus aureus* from surface water samples from watersheds with and without commercial hog operations (CHOs). Furthermore, this study considers antibiotic resistant *S. aureus* broadly rather than exclusively focusing on methicillin resistant *S. aureus* (MRSA). We identified isolates with and without livestock-associated markers, indicating that multiple sources may be contributing *S. aureus* to the environment. While this study does not directly identify sources of contamination, sources could include septic tanks, wild animals, humans, and or insect and rodent vectors (Hatcher et al., 2016; Viau et al., 2011; Wardyn et al., 2012). As all *S. aureus* isolates from background sites did not have *scn*, it is unlikely that human contamination, such as from septic tanks, is contributing *S.* aureus to surface water at these sites.

No *S. aureus* was present between February 2017 and April 2017 when average water temperature at sites was 17.2° C and ranged between 13.7° C and 20.7° C. This was higher in both range and average than winter as whole (8.9°C - 16° C; 12.4° C) when *S. aureus* was still recoverable. This lack of *S. aureus* from February 2017 and April 2017 is discordant with Levin-Edens et al. (2011), as it falls within the survival limits of *S. aureus* in freshwater. However, it has been suggested that *S. aureus* survives differently in varying climates and further research is needed to determine the survival of *S. aureus* from warmer regions such as North Carolina in fresh water (Levin-Edens, Bonilla, Meschke, & Roberts, 2011).

4.2 Methicillin Resistant *S. aureus* and Antibiotic Resistance

One unique *S. aureus* isolate originating from a CHO site showed antibiotic resistance to penicillin outside of the multidrug resistant *S. aureus* (MDRSA) identified. The remaining 15 unique isolates from both CHO and background sites were susceptible to all antibiotics tested. This finding suggests that antibiotic resistant *S. aureus* was not prominent in the surface waters tested by this study, but occurred episodically in watersheds housing CHOs.

No methicillin resistant *S. aureus* (MRSA) was identified in this study. Previous studies have solely focused on isolating MRSA in water systems, yet a limited number have purposely investigated methicillin susceptible *S. aureus* (MSSA). Designing experiments to isolate MRSA but not MSSA could lead to lower detection of *S. aureus* in water systems and limit the potential of isolating and documenting MDRSA in the environment.

4.3. Multidrug Resistant *S. aureus* (MDRSA)

Multidrug resistant *S. aureus* (MDRSA) was isolated on one occasion from a CHO site that exhibited signs of a hog lagoon spill or discharge. Physical and chemical conditions of the site were abnormal on the sampling date MDRSA was found and suggested a presumptive hog lagoon spill. Water appeared pink in appearance, measured conductivity was 3.2 times that of the average for that site (122.2 SPC compared to 400.5 SPC), the water was exceptionally odorous, and this site produced the highest most probable number (MPN) of *S. aureus* identified by this study. Additionally, all MDRSA isolates were *scn* negative and resistant to tetracycline, two different livestock-associated (LA) markers. MDRSA isolates included three *spa* types. One unique MDRSA isolate had *spa* type t2315 and three had *spa* type t337. These *spa* types belong to CC9 and CC398 respectively, both of which have a known association with hogs and are considered a LA marker (Skallerup et al., 2015). The final *spa* type identified in MDRSA

isolates, t17704, was newly identified by this study and therefore has no previous associations. Four unique isolates from this event were positive for all three LA markers. No other isolates from this study presented with LA *spa* types, nor were positive for all three LA markers.

MDRSA was only found on this occasion during the span of our study and was not present again at this site. This finding suggests that MDRSA can be found episodically in surface water in watersheds containing CHOs. Transfer of antibiotic resistant bacteria from CHOs and other concentrated animal operations (CAFOs) has been documented through nasal carriage in workers, air transport, and run off of applied feces to fields (Hatcher et al. 2017; Copeland 2003; West et al. 2011; Sapkota et al. 2007; Schulz et al. 2012). However, as sampling was limited to twice per season, it is notable that this presumptive lagoon spill and MDRSA was captured by our study. This indicates that hog lagoon contamination could be occurring more frequently, and that more intensive monitoring of this site and of other watersheds could be warranted.

4.4 Study Limitations

There is not one unified method or preferable media for isolation of *S. aureus* from environmental samples. Through a short pilot study comparing different enrichment methods and medias for isolation of *S. aureus*, enrichment in 6.5% NaCl + Muller Hinton broth for 48 hours followed by plating on CHROMagar™ Staph Aureus (Chromagar, Springfield, NJ) was chosen as the preferable method. The limitations of CHROMagar™ Staph Aureus (Chromagar, Springfield, NJ) in environmental applications are well documented (Goodwin and Pobuda 2009; Hatcher et al. 2016; Levin-Edens et al. 2012, 2011). Additionally, the manufacturer's instructions provide the vague and widely applicable description of "purple to mauve" to describe *S. aureus* colonies. We suggest the more detailed criteria determined by this study for defining the appearance of *S. aureus* isolates on CHROMagar™ Staph Aureus (Chromagar,

Springfield, NJ) in order to prevent a high level of false positives: shiny, opaque, bright purple to dark magenta colonies with a slightly raised circular form.

While selective for *S. aureus* in clinical samples, CHROMagar™ Staph Aureus (Chromagar, Springfield, NJ) was not reliably selective for *S. aureus* in environmental samples, even after enrichment. Mixed colonies were common and competition with other organisms may have lowered the recoverable number of *S. aureus* isolates. Thus, *S. aureus* may have been present in samples, but proved unrecoverable due to the complications and limitations of the chosen media. As up to five colonies per plate were chosen for analysis by this study, not all *S. aureus* found was analyzed for antibiotic resistance and LA markers. However, as the majority of isolates collected from an enrichment tube presented with identical *spa* types and antibiotic resistance patterns, it can be inferred that only minimal characterization was missed by this constraint.

All isolates fitting the morphology description for *S. aureus* on CHROMagar *S. aureus* plates were secondarily confirmed on BBL™ Mannitol Salt Agar (MSA) (BD, Sparks, MD). This media further reduced the number of false-positives found, as not all presumptive isolates were positive for mannitol fermentation on MSA agar. When genetically confirmed, all presumptive isolates negative for mannitol formation were also negative for the *spa* gene and therefore were not *S. aureus*. Based on this study, use of MSA in addition to CHROMagar™ Staph Aureus is highly recommended by this study for the isolation of *S. aureus* from environmental samples.

4.5 Generalizability

This study highlights the presence of *S. aureus* in watersheds with and without CHOs, and reveals episodic presence of MDRSA in CHO sites. While Hatcher et al. (2016) identified

the potential to identify MRSA in watersheds with CHOs, no comparison was made with watersheds without CHOs. This study builds on the knowledge that MRSA can be found in surface waters near CHOs, and adds that MSSA can also be identified in surface waters from watersheds with and without CHOs. This study further expands the work of Hatcher to include isolation of *S. aureus* resistant to many different classes of antibiotics, not only MRSA. Finally, our results show that MDRSA can be present episodically in surface waters from watersheds with CHOs, indicating a potential source of contamination from CHOs.

CHAPTER 5: CONCLUSION

This research suggests that livestock-associated, multidrug resistant *S. aureus* (MDRSA) can be present in surface water near CHOs episodically, and most likely occurs when waste management practices fail. This finding is of critical interest to multiple stakeholders and community members. While CHO farmers should ensure proper disposal of waste is occurring on their farms to prevent the spread of MDRSA into surface waters, larger management contractors and corporations employing the CHO farmers should also ensure that their businesses are implementing and enforcing best practice waste management. Furthermore, waste management practices should be improved and implemented such that they consider the potential for contamination of nearby surface waters with multidrug resistant bacteria. Consumers should also consider their role in purchasing from companies who routinely use antibiotics for animal production, as this practice yields consequences for antibiotic resistance, particularly for zoonotic microbes such as *S. aureus*. Regulatory agencies should consider using traditional monitoring or alternative markers such as conductivity tests to determine when contamination of surface water has occurred and to highlight sites in need of interventions. Future work regarding the presence of *S. aureus* in the environment should more broadly include isolation of *S. aureus* and not just that of methicillin resistant *S. aureus* (MRSA). This technique can produce comprehensive results and insights into microbial contamination in waterways that would otherwise be missed due to early stage selectivity. Finally, increased cooperation between contractors, regulatory agencies, researchers, and CHOs can increase our knowledge of microbial contamination of nearby surface waters and act to prevent it.

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