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Isolation of methicillin-resistant coagulase-negative *Staphylococcus* (MRCoNS) from a fecal-contaminated stream in the Shenandoah Valley of Virginia

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Isolation of Methicillin-Resistant Coagulase-Negative *Staphylococcus* (MRCoNS) from a Fecal-Contaminated Stream in the Shenandoah Valley of Virginia

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Abstract

Staphylococcus is comprised of 41 known species, of which 18 can colonize humans. Despite the prevalence of infectious *Staphylococcus* within hospital settings and agriculture, there are few reports of *Staphylococcus* in natural bodies of water. A recent study by the US Food and Drug Administration found substantial contamination of poultry and other meats with *Staphylococcus*. We hypothesized that intensive farming of poultry adjacent to streams would result in contaminated runoff, resulting in at least transient occurrence of *Staphylococcus* spp. in stream waters and sediments. In this study, we sought to determine whether *Staphylococcus* occurs and persists within Muddy Creek, a stream located in Hinton, Virginia that originates at the Appalachian Mountains of Virginia and runs through various agricultural fields and adjacent to a poultry processing plant in the central Shenandoah Valley. Five different *Staphylococcus* spp. were detected in water and sediment from Muddy Creek. Mannitol Salt Agar (MSA) was used to isolate eleven *Staphylococcus* from both water and sediment. These isolates were Gram-positive, catalase-positive, and oxidase-negative cocci that were capable of fermenting mannitol. In addition, a method for screening putative staphylococci species from stream water and sediment was developed. Ten out of the eleven tested isolates were oxacillin resistant (now used to identify phenotypic methicillin-resistance) using a Kirby Bauer disc diffusion test. Furthermore, the isolates were susceptible to trimethoprim/sulfamethoxazole, tetracycline, and gentamicin while two of the isolates were resistant to erythromycin. Additionally, the BOX-PCR repetitive sequence fingerprinting method verified the presence of nine different strains among the isolates. Sequencing of the 16S rRNA gene identified five of the isolates as *Staphylococcus equorum.* The Biolog identification protocol further identified the remaining isolates as *Staphylococcus xylosus*, *Staphylococcus lentus, Staphylococcus succinus,* and *Staphylococcus sciuri*. Finally, polymerase

chain reaction amplification (PCR) confirmed that ten of the eleven isolates harbored the *mecA* gene known to confer methicillin-resistance. Overall, the occurrence of coagulase-negative staphylococci (MRCoNS) in stream water and sediment represents a potential environmental and human health concern.

Introduction

Staphylococcus is a bacterial genus comprised of 41 known species, of which 18 possess the ability to colonize humans (Schaechter, 2009). All staphylococci are Gram-positive cocci with an approximate diameter of 0.7-1.2 μm. Growth patterns occur as single or paired cells in liquid media and form grape-like clusters in solid media. Furthermore, staphylococci express the catalase enzyme responsible for the decomposition of harmful hydrogen peroxide within the cell. In general, staphylococci most commonly inhabit animals on the skin and the mucous membranes. However, *Staphylococcus* spp. have occasionally been isolated from other sources including soil, water, and food (Faria *et al.*, 2009). Microbiologists generally divide the genus based on the presence or absence of the coagulase enzyme, which is responsible for converting fibrinogen to fibrin (Leboffe, 2006). For example, a laboratory coagulase test helps to distinguish between *S. aureus* (coagulase-positive) and *S. epidermidis* (coagulase-negative), both of which are common species known to inhabit humans.

Staphylococcus species – and particularly *S. aureus* – are responsible for potentially fatal infections of the epidermal tissue in humans. Additionally, members of this genus are known to cause endocarditis and septic shock. *Staphylococcus* release superantigens – a class of antigens known to activate T-cells and cause cytokine release – leading to fever, capillary leak, and multiorgan failure (Lowy, 1998). *Staphylococcus* species commonly contain regulatory genes, such as *agr*, which modulate the expression of extracellular proteins known to contribute to the organism's virulence (Lowy, 1998). As the stages of infection progress, these genes are regulated in order to attach to host cells for colonization purposes or to spread to adjacent tissues (Lowy, 1998).

In particular, *S. aureus* may acquire the *mecA* gene – responsible for methicillin-resistance – through conjugation. Methicillin-resistant *S. aureus* (MRSA) was first isolated in

1961, one year after The Beecham Group developed methicillin from a penicillin derivative (Soge, 2009). Initially, the majority of MRSA cases were hospital-acquired, but soon after in 1980, clinicians recorded the first notable cases of community-acquired MRSA (CA-MRSA)(Huang, 2006). MRSA, similar to other staphylococcal infections, cause infection via transmission from person to person through human-to-human interactions. However, MRSA infections are exceptionally difficult to treat due to the resistance to methicillin and the lack of other effective treatment options. The problem continues to grow as recent findings show that MRSA strains are resistant to multiple antibiotics such as clindamycin and mupirocin (Wang *et al*., 2012). With growing numbers of MRSA cases both in health care facilities and in the community at large, research on this deadly bacterium has increased.

Although taxonomically and biochemically distinct from *S. aureus*, *S. epidermidis* is also an opportunistic pathogen and represents an important species within the coagulase-negative staphylococci (CoNS). With the discovery of a growing CoNS population able to infect humans, the presence or absence of coagulase no longer distinguishes between pathogenic and non-pathogenic *Staphylococcus* (Schaechter, 2009). Infections from CoNS commonly include skin infections, urinary tract infections, and arthritis (Bhargava, 2012). Further concern has arisen as methicillin-resistant coagulase-negative staphylococci (MRCoNS) have been isolated from various types of livestock (Bhargava, 2012). In addition to their own increased potential for resisting antibiotics used to treat CoNS infection, MRCoNS possess the capability to serve as reservoirs of antimicrobial resistant genes that can horizontally transfer to other pathogenic bacteria, including *S. aureus* (Bhargava, 2012).

Despite the prevalence of infectious *Staphylococcus* within hospital and even some agricultural settings, few reports exist in the literature regarding the isolation and

characterization of *Staphylococcus* from natural bodies of water (Soge, 2009). In the study performed by Soge, *S. aureus* was isolated from US West Coast marine beaches, which suggests that humans are able to shed *S. aureus* in water environments and that *S. aureus* possesses the necessary adaptive properties to survive, at least for a time, in salt water. Although *Staphylococcus* has been found in and on livestock (Weese 2010), there are to date no known reports of *Staphylococcus* occurring in agriculturally impacted bodies of fresh water. The presence of contaminated runoff from nearby farms may introduce a novel population of transient yet pathogenic bacteria. Due to growing reports of *Staphylococcus* isolation from marine and livestock bacterial populations, agriculturally impacted streams represent a potential new site for contamination with staphylococci. Implications of these interactions include contamination of other waterways as well as of human food sources. The occurrence and especially persistence of *Staphylococcus* in stream water and sediment may also lead to an increased potential for antibiotic resistance gene transfer to and from native bacteria in these systems.

The aims of this study were to develop a rapid isolation method to determine whether *Staphylococcus* was present in Muddy Creek, to identify the genus and species of each isolate, and to determine the susceptibility of *Staphylococcus* isolates to a range of relevant antibiotics, including oxacillin. This study represents the first reported isolation of MRCoNS from agriculturally impacted bodies of freshwater. Specifically, ten methicillin-resistant coagulase-negative staphylococci were isolated from Muddy Creek. Although it is not yet known how representative this result is even of streams in the Shenandoah Valley, the presence of methicillin-resistant *Staphylococccus* in Muddy Creek has troubling implications for the possible

spread of resistance genes to and from native and introduced populations of Gram-positive bacteria.

Materials and Methods

Sample collection. Sediment samples were collected from Muddy Creek located in northwestern Rockingham County, Virginia. Muddy Creek flows from the foothill of the Appalachian Mountains of Virginia into the Shenandoah Valley. The creek was chosen for this study as it is heavily impacted by fecal runoff as a result of grazing cattle, poultry plant runoff (site of "Muddy Creek" samples), and farm use. The creek empties into the Dry River and North River, a tributary to the Shenandoah River. The creek ranges from a depth of 0.10 to 0.25 m and an average width of 2.5 to 5.0 m. Samples of fine sediment were collected in sterile 50 mL Falcon™ conical tubes (Fisher Scientific, Pittsburgh, PA). The top sediment layer was brushed away by gloved hand and the tube was inserted into the sediment. Water samples were collected in areas of standing water by inserting a sterile 50 mL Falcon™ conical tube approximately 5 cm under the surface. The collected samples were transported to the lab on ice and refrigerated until time of use. Samples were processed no later than 24 hrs after collection.

***Staphylococcus* isolation from sediment and water.** A mixture of 95 mL of 0.1% sodium pyrophosphate solution was applied to 5 g (wet weight) of sediment sample and mixed thoroughly for 30 s to release the cells adhering to sediment (Holben et al., 1988). Afterwards, volumes of 100 μL and 1 mL of the pyrophosphate-bacteria solution were pipetted directly onto previously prepared Mannitol Salt Agar (MSA) plates. In addition, approximately 100 mL of the water sample was filtered through a 0.5-micrometer vacuum filtration apparatus and then the filter was applied directly to an MSA plate. Plates were placed in the 37ºC incubator for 24-48 hrs. Per liter, MSA contains 7.5% sodium chloride to select for halotolerant bacteria. It can also differentiate between mannitol fermenters (phenol red in the agar turns yellow) and non-mannitol fermenters (no color change).

Isolation and biochemical tests. Initially, colonies from MSA plates were selected and Gram stained. However, due to the large number of Gram-positive rods on the MSA plate, a simple stain was alternatively used to first identify cellular morphology before Gram-status. Isolates were transferred to a microscope slide, mixed with a drop of water, and heat fixed. Crystal violet dye was then added to the slide for 60 s and rinsed with water. Cellular morphology was examined under 1000X magnification using immersion oil (Leboffe, 2006). Cells exhibiting coccus morphology were then Gram stained. Heat fixed emulsions were stained using crystal violet, iodine, ethanol, and safranin (Leboffe, 2006). After drying, the slides were examined at 1000X magnification. Gram-positive cocci were then selected for further biochemical tests. It was determined that colonies of Gram-positive cocci could be easily distinguished on MSA plates, as they produced milky-white colonies with a diameter of roughly 0.2 cm. These colony characteristics were later used for rapid identification of putative *Staphylococcus* isolates.

The KOH test was used to validate the Gram stain results. One drop of 3% KOH (potassium hydroxide) was placed onto a microscope slide. Bacteria were added to the KOH and mixed for 60 s to observe the appearance of a mucoid string (Murray, 1999). A catalase slide test was performed to identify if the isolates produced the enzyme catalase, which breaks down hydrogen peroxide. For each isolate, a large number of cells were transferred to a microscope slide. One drop of hydrogen peroxide was placed onto the cells and observed for the presence of bubbles (Leboffe, 2006). An oxidase test was performed to identify if the isolates produced the enzyme cytochrome c oxidase. A visible mass of growth was transferred onto a BBL™ DrySlide™ (BD BBL™, Franklin Lakes, NJ) using a sterile toothpick. After 20 s, the cells were observed for a color change to blue (positive result) (Leboffe 2006). Coccus-shaped isolates from

the mixed MSA plate that were Gram-positive, catalase-positive, KOH-negative, and oxidase-negative were purified on Tryptic Soy Agar (TSA) plates.

A coagulase test was also performed on each isolate in order to classify each putative *Staphylococcus* as either coagulase-positive or -negative. A coagulase test assesses an organism's ability to coagulate rabbit plasma through the use of the coagulase enzyme. Rabbit plasma was mixed with a heavy inoculum of bacteria and the tubes were incubated for 24 hrs at 37ºC. Following incubation, the tubes were examined for coagulation of the plasma (Leboffe, 2006).

Amplification and sequencing of the 16S rRNA gene. In order to identify the genus and species of the isolates, a 16S rRNA PCR was performed. The forward and reverse primers used were Bac8f and Univ1492r (Table 1), both at a final concentration of 0.125 µM. A small number of cells were taken from isolated colonies on TSA plates and added to 10 µL of double distilled water (ddH_2O) in 0.2 mL PCR tubes. The cell suspension was placed into a Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) for 15 min at 95ºC to lyse the cells. Ten microliters of Epicentre premix I (Epicenter Technologies, Madison, WI; containing 100 mM of Tris-HCl (pH 8.3), 100 mM KCl, 400 µM of each dNTP, 7 mM of $MgCl_2$, and 8X FailSafe PCR Enhancer), 20 µL of primer mix, 5 µL of the Failsafe enzyme mix (Epicentre), and 5 µL of cell lysate were mixed together. All PCR tubes were placed into the Bio-Rad C1000 Touch™ Thermal Cycler and underwent the following automated amplification cycles: 95ºC for 5 min and then 35 cycles of 95ºC for 60 s, 50ºC for 30 s, and 72ºC for 90 s (Temple, 2008). After amplification, 10 µL of amplified DNA were visualized using an agarose gel as described below. Visible bands at approximately 1500 bp represented the amplified 16S rRNA PCR product. Successfully amplified isolates were sent to MWG Operon (Huntsville,

Alabama) for Sanger sequencing of the 16S rRNA gene using the Bac8f primer.

Table 1. Primers used in this study. "N/A" denotes that the BOX primers amplify variable intervening regions between conserved BOX sequences, which may result in varied product sizes and regions amplified.

Primer Name	Gene or region Amplified	Nucleotide Sequence 5'-3'	Expected Product Size (bp)
Bac8f	16S rRNA	AGAGTTTGATCCTGGCTCAG	1500
Univ1492r	16S rRNA	GGTTACCTTGTTACGACTT	1500
BOX	N/A	CTACGGCAAGGCGACGCTGACG	N/A
mecA_fwd	*mecA*	TGAAGTAGAAATGACTGAACGTCCG	1632
mecA_rev	*mecA*	TCTGCAGTACCGGATTTGCC	1632
mecA2_fwd	*mecA2*	GGAGACCAGACGTAATAGTACCTGG	1559
mecA2_rev	*mecA2*	AGCATTATAGCTGGCCATCCC	1559
mecA400_fwd	*mecA*	AACGTTGTAACCACCCCAAGA	407
mecA400_rev	*mecA*	GTTCTGCAGTACCGGATTTGCC	407
mecA2_500_fwd	*mecA2*	GCCGTGTTTATCCATTGAACGAAGC	496
mecA2_500_rev	*mecA2*	TGGGTTGAACCTGGTGATGTAGTG	496

Identification using the Biolog Microbial ID System. Further identification was performed using the The Biolog Microbial ID System (Biolog, Hayward, CA) according to the manufacturer's protocol (Biolog, 2008). A colony from a pure culture growing on TSA was selected and transferred to a new TSA plate and incubated at 33ºC for 24 hrs. The Biolog protocol recommends using Biolog Universal Growth media (BUG), but states that TSA is a valid substitute (Biolog, 2008). Cotton-tipped inoculator swabs were used to select an isolated

colony approximately 3 mm in diameter. A Biolog turbidimeter was calibrated and blanked using fresh inoculating fluid (IF), which contains 0.03% Tween 40 and 0.25% Gellan Gum (Biolog, 2010). The swab containing bacteria was inserted into the tube containing IF and the swab tip was rubbed against the bottom of the tube to release the bacteria into the liquid. A Vortex shaker was used to create a homogenous cell suspension. The inoculated tube was adjusted to a percent transmittance of 90-98% T. The adjusted cell suspension was poured into a multichannel pipet reservoir and 100 µL of cell suspension was added to each well of a prepared 96-well plate using an 8-channel repeating pipettor. A Biolog GEN III MicroPlate was used to analyze each isolate through the use of 94 phenotypic tests, which creates a "phenotypic fingerprint" for identifying the genus and species. The inoculated MicroPlate was covered with a lid and then incubated for 18 hrs at 33ºC. Afterwards, the plate was placed into the OmniLog reader and Biolog's Microbial Identification Systems software (OmniLog® Data Collection) was used to identify each isolate. Throughout the experiment, a positive control (*Staphylococcus epidermidis*) was used to control for each step of the protocol.

Repetitive sequence PCR for strain differentiation. Isolates were differentiated using the BOX-PCR repetitive sequence fingerprinting method. This procedure works by amplifying variable regions between a BOX sequence that is conserved amongst most bacterial species in order to differentiate between possible strains. The procedure was carried out essentially according to the protocol described by Rademaker *et al*. (1998). A small number of cells were taken from isolated colonies on TSA plates and added to 10 µL of double distilled water (ddH_2O) in 0.2 mL PCR tubes. The cell suspension was placed into a Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) for 15 min at 95ºC to lyse the cells. In a separate 0.2 mL PCR tube, 13 pmol of BOX primer (Table 1), 0.1 µl Failsafe enzyme mix (Epicentre),

0.6 µl of sterile ddH20, 5 µL of Premix G (Epicentre), and 3 µL of crude lysate were added. The sequence of the BOX primer is listed in Table 1. PCR was performed using the following amplification program: 95ºC for 2 min and then 30 cycles of 94ºC for 3 s, 92ºC for 30 s, 50ºC for 60 s, with a final extension step at 65ºC for 8 min. After amplification, 4 µL of amplified DNA were visualized using an agarose gel as described below. The resulting BOX-PCR "fingerprints" were compared between isolates based on banding patterns.

Detection of antibiotic resistance. A Kirby-Bauer test was used to assess the antibiotic susceptibility of isolates (Bauer *et. al*, 1959). A lawn of bacteria was made on Mueller-Hinton agar and antibiotic discs (BD BBL™ Sensi-Disc™ Antimicrobial Susceptibility Test Discs, Franklin Lakes, NJ) were placed in different sectors of the plate. Trimethoprim/sulfamethoxazole (1.25 µg/23.75 µg), tetracycline (30 µg), gentamicin (10 µg), erythromycin (15 µg), and oxacillin (1 µg) discs were used for this experiment. The plates were incubated for 24 hrs at 37ºC. After incubation, the diameter of the zone of inhibition was measured and compared to standard measurements to determine whether the isolate was resistant or susceptible.

PCR amplification of *mecA*. A multiplex PCR technique was used to detect whether isolates harbored the *mecA* gene. Isolates were transferred to 0.2 mL PCR tubes containing 15 µL of ddH_2O using an inoculating needle. In order to lyse the cells, the cell solution was exposed to 95ºC for 15 min. Afterwards, 1 µL of DNA lysate was transferred to a 0.2 mL PCR tube along with 10 µL of Promega PCR Master Mix (Promega Corporation, Madison, WI; containing 50 units/mL of *Taq* DNA polymerase, 400 µM of each dNTP, and 3 mM of $MgCl_2$), 8 µL of ddH_2O, and a multiplex primer solution with a final concentration of 0.05 µM. The multiplex primer solution contained 0.1 µM of each of the following primers: mecA1 forward/reverse, mecA2 forward/reverse, mecA400 forward/reverse, and mecA2-500 forward/reverse in 40 µL of ddH_2O

(Kondo *et al.*, 2007). The 5'-3' nucleotide primer sequence for each primer is listed in Table 1. The primers mecA1 and mecA2 amplify large regions of the *mecA* and *mecA2* gene respectively. The primers mecA400 and mecA2-500 amplify small regions of the *mecA* and *mecA2* gene respectively. All PCR tubes were placed into the Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) and underwent the following automated amplification cycles: 94ºC for 2 min and then 30 cycles of 94ºC for 2 min, 57ºC for 60 s, and 72ºC for 2 min, with a final extension step of 2 min at 72ºC. After amplification, 10 µL of amplified DNA were visualized using an agarose gel as described below. DNA was isolated from the N315 strain of methicillin-resistant *Staphylococcus aureus* (MRSA) and was used as a positive control.

Gel electrophoresis of PCR products. Following PCR, amplified DNA was loaded into a 1% agarose gel with 2 µL of 6X Blue/Orange Loading Dye (Promega Corporation, Madison, WI) and electrophoresed at 80 V for 2 hours. A 1 kb Fisher BioReagents™ exACTGene™ DNA Ladder (Fisher Scientific, Pittsburgh, PA) was used. The gel was stained using a 0.5% ethidium bromide solution for 20 min. The gel was then destained with ddH_2O for 10 min. Photographs of the gel were taken with a Bio-Rad ChemiDoc™ XRS+ System (Bio-Rad Laboratories, Hercules, CA) and analyzed using the Image Lab™ Software.

Results

Sample collection and processing. In January 2013, two sediment samples and two water samples were collected from Muddy Creek in Hinton, Virginia. Sediment samples were named "sediment up" and "sediment down" according to the collection site. "Sediment up" samples were collected closer to the drainage pipe of the poultry processing plant whereas "sediment down" samples were collected downstream from the waste pipe. A similar naming system was applied to the water samples. Additionally, a second sampling was performed in March 2013 with two sediment and two water samples collected.

After performing sediment cell release and water filtration, cells from the released cell solution and from the water filter were plated onto MSA plates in order to selectively isolate halotolerant bacteria such as staphylococci. A greater diversity of colony number, shape, size, and morphology was found on the sediment plates (both the winter and spring samples) compared to water.

Isolation and identification of *Staphylococcus*. Colonies were originally chosen at random to determine cell morphology (cocci) using a Gram stain technique. However, the majority of the colonies that grew on the MSA plate were Gram-positive rods. Therefore, a simple stain was performed to first screen for cellular morphology and select all cocci for further tests. After numerous rounds of simple staining, a general correlation between coccus cellular morphology and colony morphology was determined. All of the elevated milky-white colonies roughly 0.2 cm in diameter were determined to be cocci after performing a simple stain (Figure 1). Selecting colonies on MSA based on colony morphology created a rapid and simple initial screening method for putative staphylococci.

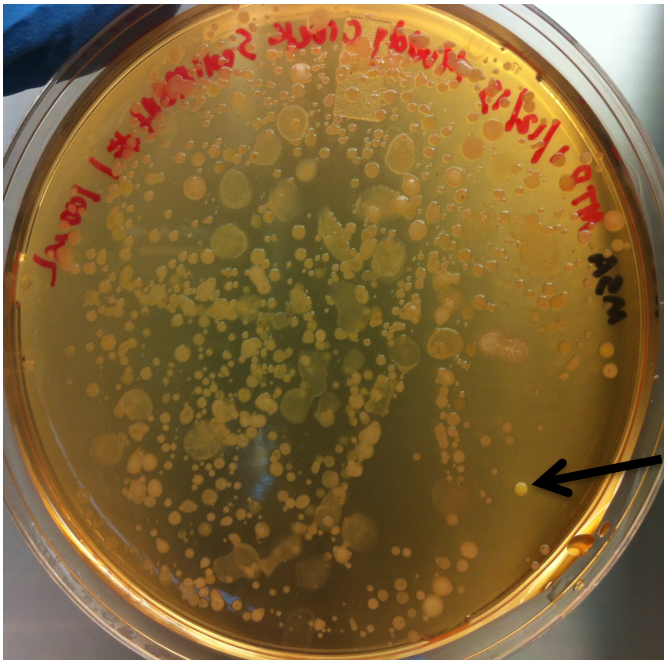

Figure 1. Example of colony morphology used to isolate putative *Staphylococcus* sp.. The black arrow points to a colony selected based on its milky-white appearance and ca. 0.2 cm diameter.

All of the elevated milky-white colonies roughly 0.2 cm in diameter (11) were selected and were found to be coccus-shaped. The cells clustered in "grape-like" patterns, which is common to the genus *Staphylococcus*. Ten of the eleven putative *Staphylococcus* (PS) were isolated from the stream sediment (Table 2). A Gram stain, KOH, catalase, oxidase, and coagulase test was performed on each of the eleven isolates for further identification. All eleven isolates were Gram-positive, KOH-negative, catalase-positive, oxidase-negative, and coagulase-negative cocci.

Table 2. Locations and collections dates of eleven *Staphylococcus* isolates collected from Muddy Creek in Hinton, Virginia.

Isolate	Date Collected	Source	Location of Collection
PS1	January 12, 2013	Sediment	Upstream near poultry plant waste pipe
PS2	January 12, 2013	Water	Downstream of poultry plant waste pipe
PS3-11	March 29, 2013	Sediment	Upstream near poultry plant waste pipe

The 16S rRNA genes of the isolates were amplified for sequencing using PCR; however, only PS6-8, 10, and 11 produced an amplified 16S rRNA band at approximately 1500 bp. Therefore, these isolates were submitted for partial sequencing of the 16S rRNA gene. Sequences were compared to 16S rRNA genes in the nr (Non-redundant GenBank CDS translations) database for identification using the National Center for Biotechnology Information's BLAST® program (Basic Local Alignment Search Tool). By inserting the sequence of nucleotides for the 16S rRNA gene, the BLAST system is able to compare the sequence from an isolate to a database of known sequences. The database generates a bit score and an expect value (E-value) to determine if the match is statistically significant (Altschul *et al.*, 1997). Bit scores are based on identical alignments as well as gaps in the sequence whereas E-values describe the number of expected matches generated by chance to a random database of the same size. Thus, a high bit score and low E-value points to statistically significant matches. Table 3 summarizes the results of the BLAST search by showing the top two matches for each isolate tested as well as the bit score, E-value, percent match, and GenBank accession number.

Table 3. Summary of BLAST search results for the 16S rRNA nucleotide sequences from isolates PS6-8, 10, and 11. The top two matches based on bit score are listed for each isolate.

Isolate	Description of 16S rRNA Gene	Max Bit Score	E-value	Percent Match	Accession Number
PS6	*Staphylococcus sp. SB10-23*	1622	0.0	96%	GU595329.1
	Staphylococcus equorum strain SCSGAB0145	1616	0.0	96%	JX315320.1
PS7	*Staphylococcus sp. SB10-23*	1646	0.0	95%	GU595329.1
	Staphylococcus equorum strain SCSGAB0145	1640	0.0	95%	JX315320.1
PS 8	*Staphylococcus equorum strain C4052*	1598	0.0	96%	KF439737.1
	Staphylococcus strain C4044	1598	0.0	96%	KF439736.1
PS 10	*Staphylococcus sp. SB10-23*	1668	0.0	96%	GU595329.1
	Staphylococcus equorum isolate EO2001	1663	0.0	96%	AY741060.1
PS 11	*Staphylococcus sp. SB10-23*	1733	0.0	98%	GU595329.1
	Staphylococcus equorum subsp. *equorum*	1727	0.0	98%	AB735696.1

The remaining isolates (PS1-5), as well as PS6 and PS7 were identified using the Biolog Microbial ID System (Biolog, 2010). By exposing each isolate to 94 different biochemical tests all within one 96 well plate, a unique fingerprint is generated for each species of bacteria through colorimetric changes. Biolog's Microbial Identification Systems software (OmniLog® Data Collection) then matches the optical density (OD) of the color in each well to a known database of intensities to identify the unknown organism. After reading a plate, a number of possible organisms appear along with a PROB, SIM, and DIST value. The PROB value allows for a comparison of Biolog's identification to other systems using similar identification calculations.

A SIM value is used to determine the accuracy of the identification with a "1" being a perfect match and "0" meaning no match (Biolog, 2010). DIST represents the approximate number of mismatches between the results of the unknown organism and information from the database of the known organism proposed to be a match. Biolog's User Guide explains that answering the following three questions "yes" ensures a confident match for the top listed organism. First, do the top listed organisms belong to the same or similar genus/genera? Second, for the number one identification, is the SIM value greater than 0.5? Third, is the DIST value for the number one identification at least two distance points away from the second choice (Biolog, 2010)?

PS2 was unable to be identified due to the low SIM value and therefore resulted in a "No ID" result. The remaining isolates were all identified with a SIM value greater than 0.5 (Table 4). Furthermore, the ΔDIST is the DIST value of the first matched organism subtracted from the second organism and all of the identified isolates were less than two distance points (Table 4). If the top-listed identifications all belong to the same genus, then the confidence level of identification is strengthened. The Biolog Microbial ID System produced top-listed matches from the genus *Staphylococcus* for PS 1, 4, and 6. PS 3, 5, and 7 were identified as *Staphylococcus* spp. for the top match but subsequent matches showed different genera consisting of Gram-positive rods. *Staphylococcus epidermidis* was used as a positive control and the Biolog software identified the strain as *Staphylococcus epidermidis* with a PROB, SIM, and ΔDIST of 0.995, 0.783, and 3.78 respectively. All of the other top-listed matches belonged to the *Staphylococcus* genus. PS9 was unable to be identified either via 16S rRNA sequencing or Biolog and remains unidentified. However, the biochemical characteristics as well as the cellular morphology resembled all of the other isolates and therefore PS9 is likely to be an organism belonging to the genus *Staphylococcus*.

Table 4. PROB, SIM, and Δ DIST values of isolates using the Biolog Microbial ID System. ΔDIST is the DIST value of the first matched organism subtracted from the second organism.

Isolate	Identification	PROB	SIM Value	ΔDIST
PS1	*Staphylococcus xylosus*	0.788	0.561	1.164
PS2	NO ID	-	-	-
PS3	*Staphylococcus lentus*	0.562	0.562	0.173
PS4	*Staphylococcus sciuri* subsp. *sciuri*	0.815	0.667	1.036
PS5	*Staphylococcus sciuri* subsp. *sciuri*	0.653	0.653	0.156
PS6	*Staphylococcus succinus* subsp. *succinus*	0.603	0.603	0.739
PS7	*Staphylococcus equorum*	0.529	0.529	0.072

Repetitive sequence PCR for strain differentiation. After identifying the genus and species of each isolate (except for PS2 and PS9), a type of repetitive sequence fingerprinting known as "BOX-PCR" (Rademaker *et al.*, 1998) was performed on all isolates in order to differentiate between strains. This method uses a primer set that amplifies intervening regions between a conserved BOX sequence thus creating a unique banding fingerprint for different strains of bacteria. Amongst the eleven isolates, nine different banding patterns were produced (Figure 2). PS6 and PS10 produced the same pattern while PS7 and PS11 produced identical banding patterns to one another and only differed from PS6/PS10 by the lack of a band at 600 bp (Figure 2). PS8 resembled PS6/PS10 but lacked the 600 bp band and produced an additional band at approximately 680 bp. PS2-5 all produced distinct banding patterns. PS9 and PS1 were not successfully amplified, but produced distinct banding patterns to the one another and the other isolates in previous BOX-PCR attempts not pictured here. *E. coli* strain LA61 was used as a positive control. Although anomalous bands are found in the negative control lane, the banding patterns of each isolate match the fingerprints from numerous previous attempts.

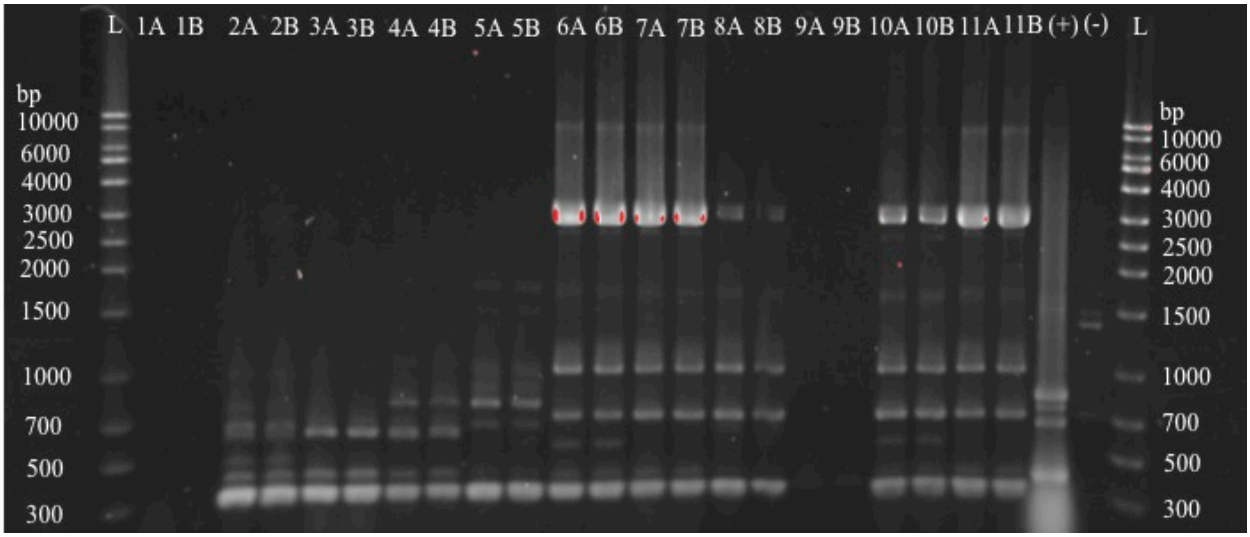

Figure 2. BOX-PCR of isolates used to differentiate isolated strains using a 1% agarose gel and ethidium bromide staining. A 1 kb Fisher BioReagents™ exACTGene™ DNA Ladder ("L") (Fisher Scientific, Pittsburgh, PA) was used as a size marker. Each isolate was labeled without the "PS" designation and with the isolate number only. Amplified products of each isolate were electrophoresed in duplicates and signified by the "A" and "B" following the isolate number. *E. coli* strain LA61 was used as a positive control (+). A negative control consisting of water and the PCR reagents was also used (-).

Antibiotic susceptibility. The Kirby-Bauer test was used to test for susceptibility to oxacillin (now used to identify phenotypic methicillin-resistance), as well as susceptibility to other antibiotics. Trimethoprim/sulfamethoxazole (1.25 μg/23.75 μg), tetracycline (30 μg), gentamicin (10 μg), erythromycin (15 μg), and oxacillin (1 μg) discs were each placed on a lawn of bacteria on Mueller-Hinton agar. The zone of inhibition diameter was measured and compared to the interpretative chart supplied by BD BBL™. All isolates (PS1-11) were susceptible to trimethoprim/sulfamethoxazole, tetracycline, and gentamicin. All of the isolates except for PS2 and PS3 were susceptible to erythromycin whereas PS2 and PS3 were resistant. Additionally, all of the isolates were resistance to oxacillin (≤17 mm) except for PS6, which exhibited a zone of inhibition diameter of 19 mm (Figure 3).

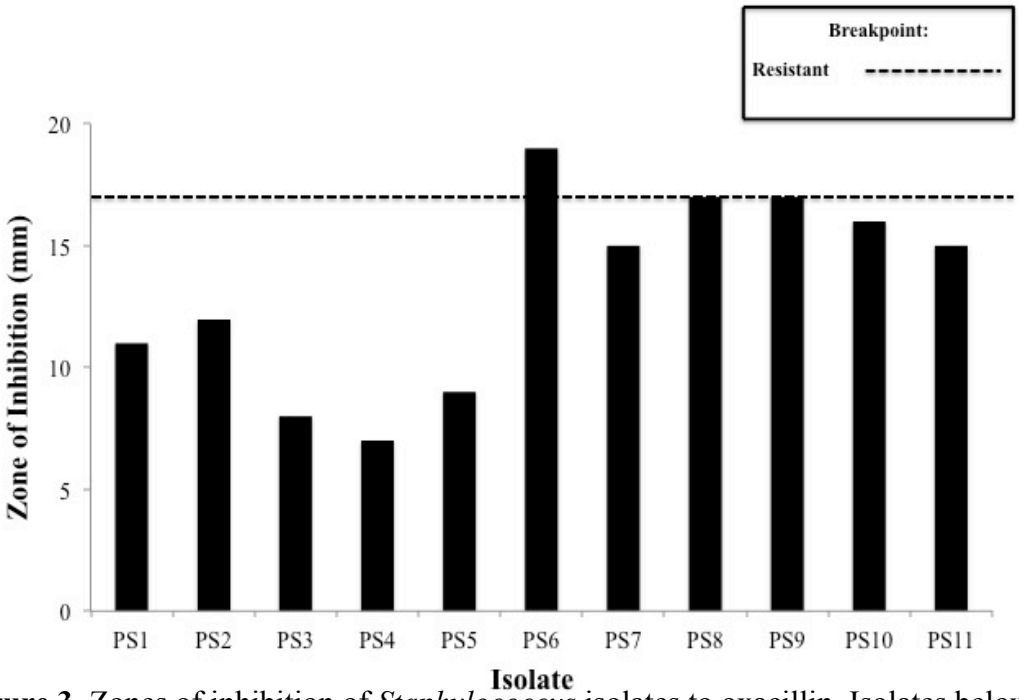

Figure 3. Zones of inhibition of *Staphylococcus* isolates to oxacillin. Isolates below the dotted line (17 mm) are resistant to oxacillin and isolates above the dotted line are susceptible. This breakpoint is according to the interpretative chart supplied by BD BBL™ for coagulase-negative staphylococci to oxacillin.

Detection of the *mecA* gene encoding resistance to oxacillin. Multiplex PCR was performed in order to detect the *mecA* or *mecA2* gene, which both confer resistance to oxacillin and methicillin. The multiplex PCR combines different primer sets to amplify different regions of the *mecA* and *mecA2* genes (Kondo *et al.*, 2007). The *mecA* primers amplify a 1632 bp region of the *mecA* gene whereas the mecA2 primers amplify a 1559 bp region of the mecA2 gene. The mecA400 primers amplify a 407 bp region of the *mecA* gene whereas the mecA2_500 primers amplify a 496 bp region of the *mecA2* gene (Table 1). All eleven isolates (PS1-11) were assessed for the presence of the *mecA* gene. All isolates except for PS2 produced a faint band at approximately 400 bp suggesting the presence of the amplified mecA400 product of the *mecA* gene. However, the image of the gel is not included because in performing the *mecA* PCR a

negative control was not used and subsequent attempts in replicating the results were unsuccessful.

Discussion

Sample collection and processing. This study aimed to isolate and to identify methicillin-resistant *Staphylococcus* from Muddy Creek, a manure-contaminated stream in Hinton, Virginia. Several studies have demonstrated the prevalence of MRSA from recreational marine and fresh water beaches, stream water, and sand samples from beaches (Levin-Edens *et al.*, 2012). However, little information exists on the occurrence of *Staphylococcus* in streams impacted by runoff from agricultural operations despite recent reports on the prevalence and ease of isolating methicillin-resistant *Staphylococcus* from farm animals (Fang *et al.*, 2014).

During January of 2013, two staphylococci (PS1 and PS2) were isolated from both water and sediment samples. Nine more staphylococci were isolated in March of 2013, all from the sediment sample. It is possible that a link may exist between temperature and the likelihood of isolating of staphylococci found in Muddy Creek. During the sampling in January, the air temperature was approximately 4.5ºC while the air temperature during the March sampling was 13ºC. Although samples were obtained from the same location, eight staphylococci were isolated from the sediment sample in March as opposed to one in January. Besides the temperature, runoff from area farms as well as the poultry plant may increase during Spring, thus contributing more waste to the stream.

Isolation and identification of isolates. Initial examination of bacteria from both stream and sediment samples growing on MSA plates revealed a large number of Gram-positive rods. Although we tested Vogel and Johnson Agar and MSA, we were not able to find a selective medium that selected against these Gram-positive rods to target staphylococci. However, after randomly testing numerous colonies that grew on MSA, it was determined that *Staphylococcus* spp. were relatively easily distinguished by their distinctive small size (0.2 cm diameter) and

milky-white color (Figure 1). Therefore, by first visually screening for these colonies on MSA plates, followed by a simple crystal violet stain for cell shape, a relatively large numbers of colonies could be examined quickly without using a great amount of time or resources. Although a number of methods have been published for screening for and isolating *Staphylococcus aureus* from natural samples, to our knowledge none exist that will do so for the entire genus (Soge, 2009).

All eleven isolates were tested for defining characteristics of the *Staphylococcus* genus. The Gram stain, catalase test, oxidase test, KOH test, and coagulase test are extremely useful in identifying members of the genus *Staphylococcus*. Each of our isolates was Gram-positive, catalase-positive, KOH-negative, oxidase-negative, and coagulase-negative. Although none of the isolates were *S. aureus* – which is coagulase positive – the threat of CoNS as human pathogens may still exist. Coagulase-negative *Staphylococcus* were originally thought of as harmless skin bacteria, but are now known to be the causative agent of 11% of all nosocomial infections (Huebner *et al*., 1999). CoNS are known to cause endocarditis, bacteremia, and urinary tract infections. Treatment methods for CoNS are continually complicated due to the rapid development of antibiotic resistance, especially in hospital settings (Huebner *et al*., 1999).

Partial 16S rRNA gene sequencing and the Biolog Microbial ID System were used to verify the identification of the isolates as members of the *Staphylococcus* genus, as well as to determine, where possible, the species of each. In addition, the BOX-PCR fingerprinting method was used to distinguish the different strains isolated, particularly since many of them were closely related to one another, as determined by the 16S rRNA and Biolog results. A portion of the 16S rRNA gene of five isolates (PS6-8, 10, and 11) was sequenced and matched to the Non-redundant GenBank database using a BLAST search. Results of the BLAST search showed that

Staphylococcus equorum was listed as one of the top matches for all of the tested isolates (Table 3). Similarly, the BOX PCR results for PS6-8, 10, and 11 all showed similar banding patterns (Figure 2).

Isolates PS6 and PS7 were the only isolates subjected to both 16S rRNA sequencing and to the Biolog tests. The Biolog results identified both the first match for PS7 and the third match for PS6 as *Staphylococcus equorum.* The top match from the 16S rRNA sequencing (*Staphylococcus sp. SB10-23)* was only itself identified to the genus level; however, the top Biolog result for PS6 was *Staphylococcus succinus* subsp. *succinus*. All of the top-rated matches for PS6 belonged to the genus *Staphylococcus*, and the SIM value for the first match was greater than 0.5 (Table 4). However, the ΔDIST (the DIST value of the first matched organism subtracted from the second match) between the first and second match was less than 2. Although two out of three of the Biolog accuracy parameters were met, the DIST value remained low. Nonetheless, Biolog notes that similar DIST values may result between top-rated matches because of the similar biochemical characteristics of the listed species (Biolog, 2010). Thus, a confident identification was made despite the low ΔDIST value. Despite the differences between the 16S rRNA sequencing and Biolog results, clearly PS6 belonged to the genus *Staphylococcus* with uncertainty as to whether the species was *equorum* or *succinus*. PS7 produced a SIM value greater than 0.5, but a ΔDIST value less than 2 and none of the top-rated generas matched *Staphylococcus*. Therefore, the Biolog results for PS7 remained inconclusive yet still matched the results of the 16S rRNA sequence, which may validate the accuracy of the Biolog experiment.

Biolog identified PS1 as *Staphylococcus xylosus*. The SIM value was greater than 0.5 and the other top matches were all *Staphylococcus* (Table 4). Also, the ΔDIST value was less than two (1.164), but was the highest ΔDIST of the isolates tested. Although not perfect, the accuracy

parameters for PS1, according to Biolog, demonstrated a confident match. None of the other isolates were identified as *Staphylococcus xylosus*. PS1's unique identification, among the other isolates, was supported by its unique BOX-PCR banding pattern (Figure 2).

PS3 was identified by Biolog as *Staphylococcus lentus*. The SIM value was greater than 0.5, but the ΔDIST value was less than two. Besides *Staphylococcus lentus*, the other top-rated matches were Gram-positive rods belonging to different genera (Table 4). However, isolate PS3 was clearly a Gram-positive coccus thus eliminating the other top matches as possibilities. This finding along with the catalase and oxidase tests supported the Biolog identification as *Staphylococcus lentus*.

PS4 and 5 were both identified by the Biolog system as *Staphylococcus sciuri* subsp. *sciuri*. The Biolog Microbial ID System calculated a SIM value greater than 0.5 and a ΔDIST value less than two for both isolates (Table 4). Additionally, all of the top-rated matches for PS4 consisted of species from *Staphylococcus* whereas those of PS5 consisted of Gram-positive rod species from other genera. Similar to PS3, PS5 was indeed a Gram-positive coccus as confirmed by a previous Gram stain. Although Biolog identified PS4 and PS5 as the same organism, the different banding patterns from the BOX-PCR suggest separate strains (Figure 2).

PS2 and PS9 were unable to be identified by Biolog or 16S rRNA gene sequencing. Despite the lack of identification, the cellular morphology and microbiological characteristics matched the other isolates that were identified as coagulase-negative staphylococci. Additionally, PS2 and PS9 each exhibited a unique BOX banding pattern relative to one another and to the other isolates (Figure 2). Therefore, if these isolates belonged to the genus *Staphylococcus*, then they may either be different strains or different species entirely when compared to the other isolates.

Description of identified species. *Staphylococcus equorum* (PS6-8, 10, and 11) was first isolated and characterized in 1984 from the skin of healthy horses (Nováková *et al.*, 2006). Later, isolates were obtained from the milk of cows with mastitis and from healthy goats (Nováková *et al.*, 2006). Isolating this organism from an agriculturally impacted stream comes as no surprise due to the fact that both goat and cow farms exist upstream from the site of collection. Additionally, worn paths are visible directly upstream from the poultry plant where cows apparently cross Muddy Creek. The Biolog Microbial ID System identified PS6 as *Staphylococcus succinus*. This species has been isolated from many sources including ripening cheese and sausage (Nováková et al., 2006). Additionally, this species was originally isolated from soil inclusions on Dominican amber (Lambert *et al.*, 1998).

S. xylosus (PS1) commonly inhabits the skin of animals such as chickens, mice, pigs, horses, and cows (Nováková *et al.*, 2006). In humans, *S. xylosus* is one of the many agents known to cause urinary tract infections and more rarely endocarditis or pneumonia (Nováková *et al.*, 2006). Similar to *S. equorum*, finding *Staphylococcus xylosus* in the sediment of an agriculturally impacted stream is not surprising. *S. lentus* (PS3) is commonly isolated from the skin of food producing animals such as chickens and cows (Schwendener *et al*., 2012). Employees who commonly work with poultry or dairy animals have been found to carry *S. lentus* and although rare, this organism may cause infection in humans (Schwendener *et al*., 2012). As the poultry plant adjacent to Muddy Creek washes down carrier trucks or dumps waste, this organism may be introduced into the water. *S. sciuri* (PS4 and 5) is mainly isolated from the skin and mucosal surfaces of pets and farm animals (Dakić et al., 2005). Additionally, common environmental reservoirs include soil, sand, and water. *S. sciuri* is also known to cause several

different complications in humans including endocarditis, urinary tract infections, and wound infections (Dakić *et al*., 2005).

Determination of methicillin-resistance. Methicillin-resistance is conferred in all known bacteria by variants of the *mecA* gene, which encodes for the penicillin-binding protein PBP2a (Kondo *et al.*, 2007). This gene is associated with the mobile genetic element SCC*mec* and commonly inserts into the chromosome of staphylococci (Kondo *et al.*, 2007). SCC*mec* elements are characterized by four distinct properties. First, the element carries the *mec* gene complex known to confer methicillin resistance. Second, the element contains a *ccr* gene complex – containing *ccr* genes and open reading frames – responsible for the mobility of the entire element. Third, direct repeat nucleotide sequences exist at both ends of the element and lastly the element inserts into the 3' end of *orfX*, an open reading frame of unknown function.

Although these common characteristics exist, structural differences within the *ccr* and *mec* components have been identified (Kondo *et al.*, 2007). For instance, four classes of *mec* gene complexes – consisting of *mecA*, regulatory genes, and insertion sequences – have been discovered (A, B, C, and D) and differ based on the arrangement and type of insertion sequence used to integrate the SCC*mec* element into the chromosome of susceptible strains. In addition, *ccr* and *mec* gene complexes combine differently on the SCC*mec* element creating genetically divergent structures. As a result, microbiologists commonly classify MRSA clones based on the SCC*mec* element makeup (IWG-SCC, 2009).

Due to the great variability in *mecA* gene complexes, we utilized a multiplex PCR technique that combined primers known to amplify large and small portions of the *mecA* gene. After performing the *mecA* PCR, bands were visible in all isolates except for PS2 at the expected product size of approximately 400 bp (small region of the *mecA* gene). Despite the lack of a

negative control, these results matched the results of the Kirby Bauer disc diffusion test in which all of the isolates were oxacillin-resistant except for PS6. However, due to the missing negative control, we are unable to make definitive conclusions at this point regarding *mecA*. PS2 was oxacillin-resistance in the disc diffusion test, but lacked the *mecA* gene. Lee *et al*. encountered this same observation when studying 3,125 staphylococci isolates from patients in hospitals. Specifically, 15 of the isolates were resistant to oxacillin, but did not have the *mecA* gene and 2 isolates were susceptible to oxacillin and harbored the *mecA* gene (Lee *et al*., 2007). The latter cases may be explained by mutations in the *mecA* promoter, which when altered may affect the levels of PBP2a translated (Chen *et al*., 2014). For instance, a G25A mutation (guanine changed to adenine 25 bases upstream of the *mecA* translation start site) was correlated to a high minimum inhibitory concentration (MIC) for oxacillin at 256 µg/mL (Chen *et al*., 2014). Other mutations have been shown to decrease or increase the MIC for oxacillin. These findings support the results for PS6 as this organism may have a mutation in the promoter region of the *mecA* complex, which lowers the MIC and causes susceptibility to the oxacillin. PS2 may harbor the *mecA* gene, but due to possible gene sequence divergence the primers used in the multiplex PCR may not bind and therefore not amplify the gene. In order to determine the reason for this anomaly, the putative *mecA* gene within PS2 could be sequenced.

Overall, in this study we isolated and identified eleven coagulase-negative staphylococci of differing species from the sediments and water of Muddy Creek. Nine of the eleven isolates were resistant to oxacillin and contained the *mecA* gene known to confer methicillin resistance. PS6 was susceptible to oxacillin in the disc diffusion test, but contained the *mecA* gene thus making ten of the eleven isolates carriers of the *mecA* gene. It is hypothesized that grazing farm animals and waste from the adjacent poultry plant are introducing these bacteria into a novel

environment. Implications of these findings might include horizontal transfer of the SCC*mec* element from such staphylococci to other (native or introduced) bacteria present in the water or sediment. Their occurrence in the stream sediment is of particular concern as it may imply that they are more persistent – or at least less transient – than bacteria found in the moving water column of the stream. Additionally, these bacteria may pose a health threat as Muddy Creek flows down stream into North River, which runs along the homes of many residents who swim and drink the water.

Future research could include determining whether or the not the SCC*mec* element, commonly found on chromosomal DNA, is plasmid-borne in these isolates and, if so, whether it is transmissible to other bacteria via conjugation. Furthermore, through isolating, amplifying, and sequencing the SCC*mec* element from these organisms, one may be able to determine information regarding the taxonomy and origin of the element itself. Finally, this study could be replicated but include sampling throughout the entire year to account for temperature and season as well as additional samples from bodies of water both affected and unaffected by agriculture. Although a direct causation does not exist between the poultry plant/farms and the presence of these staphylococci, a strong link is present and deserves further attention in order to attenuate the presence of these methicillin-resistant staphylococci.

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