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Development of an optimized protocol for the isolation of methicillin-resistance genes in *Staphylococcus aureus* bacteriophages present in the environment

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Development of an Optimized Protocol for the Isolation of Methicillin-Resistance Genes in

*Staphylococcus aureus* Bacteriophages Present In The Environment

A Project Presented to

the Faculty of the Undergraduate

College of Science and Math

James Madison University

in Partial Fulfillment of the Requirements

for the Degree of Bachelor of Science

by Taryn Katherine Boyle

May 2014

Accepted by the faculty of the Department of Biology and Integrated Science and Technology, James Madison University, in partial fulfillment of the requirements for the Degree of Bachelor of Science.

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Introduction

Antibiotics are natural and synthesized antimicrobial compounds used worldwide to protect human health, as well as a growth supplement for animals in livestock husbandry. The abuse of antibiotics, both clinically and agriculturally, has exerted selective pressures on bacteria, leading to the growing presence of antibiotic resistant strains in the environment, particularly to $\beta$-lactam penicillins (1). Specifically, methicillin-resistant *Staphylococcus aureus* (MRSA), most common in nosocomial environments, contains $\beta$-lactam antibiotic resistance genes thought to have been transferred horizontally via transduction or conjugation. Ultimately, when these resistant bacteria reach clinical settings, antibiotic resistance genes are challenged with concentrations of antibiotics and the incidence of antibacterial resistance emerges and evolves (2).

Bacteriophages (viruses that infect bacteria) constitute key vehicles for shaping the architecture of the bacterial genome, as well as acting as major vehicles for horizontal gene transfer (HGT). Phages contribute to virulence by encoding for factors that increase fitness, such as antibiotic resistance, and by their ability to move between genomes (3). It is currently believed that transduction via bacteriophages present in the environment facilitates the widespread gene transfer of these mobile genetic elements conferring antibiotic resistance (1). During bacteriophage DNA production, particles may be produced containing DNA from bacterial genomes in addition to the phage genome. This DNA can then be transferred to a subsequent host cell via HGT (4).

As a major pathogen causing illness that ranges from minor skin infections to life-threatening diseases such as pneumonia and bacteremia, *S. aureus* and its increasing incidence of methicillin resistance has long been a major concern for clinicians as well as researchers who study the origins and transfer of antibiotic resistance (5). *S. aureus* bacteriophages have been investigated
extensively; of particular importance are those temperate phages that have been regarded to be pathogenically relevant due to their ability to carry virulence genes. For example, phage 80α has been shown to mobilize a variety of superantigen-encoding pathogenicity islands (SaPIs), encoding a variety of enterotoxins and/or toxic shock syndrome toxin 1 (6).

Methicillin resistance is genetically encoded by the meca gene, which is a part of a larger mobile genetic element inserted into the Staphylococcal chromosome known as the Staphylococcal Chromosomal Cassette, (SCCmec). All MRSA strains contain the SCCmec element, which carries two essential components, a mec gene complex that encodes methicillin resistance, and ccr gene complex responsible for the mobility of the element by site-specific recombinases. Specifically, the meca gene encodes a modified penicillin-binding protein (PBP2a), which fails to bind methicillin and other β-lactams antibiotics (7). As a result, these antibiotics fail to inhibit the ability of PBP transpeptidase enzymes to cross-link the peptidoglycan polymers of the bacterial cell wall. SCCmec also contains characteristic repeating nucleotide sequences, inverted complementary sequences at both ends, and integrates into the 3’ end of the conserved open reading frame in S. aureus, ORFx. In MRSA strains, SCCmec elements are classified based on the combination of mec and ccr, which share variations of five classes of different alleles of mec and eight in ccr, and are further classified into subgroups of outside “joining-regions” (J-regions), known as J1-3. To date, at least 11 types of SCCmec have been identified (8). The exact mechanism for the transfer of SCCmec is experimentally unknown. Recently, it has been found that the cassette could be transferred piece by piece via bacteriophage transduction, which would allow the large element to become a group of normally sized mobile element (9). Further research into this mechanism of antibiotic resistance mobilization could not only elucidate the mechanism by which S. aureus acquires resistance, but also serve as a forewarning for future clinically pertinent resistance mechanisms.
The goal of our research is to discover if bacteriophages in the environment are acting as reservoirs and intermediary transfer agents of the SCC\textit{mec} element, potentially between bacteria present in streams and soils and the clinical isolates. Natural environments serve as antibiotic resistance gene reservoirs, whose viral fraction is dominated by phages that have not only shown to move between environments due to similar metabolic profiles and function (infection, replication, lysis etc.), but also to contain distinctive genetic profiles associated with manipulating host metabolisms (10). Phages have been confirmed as the most abundant beings in many natural environments such as lakes, soils, and human-managed environments such as sewage treatment plants and farms, with up to 50-60\% of detected bacteriophage particles containing bacterial genes (11). Extensive use of antibiotics in livestock operations can select for resistant bacteria such as MRSA in a similar manner of selective pressure via antibiotic usage as clinical settings. One estimate projects more than 70\% of all antibiotics and related drugs are used as feed additives in the United States (12). Furthermore, there is increasing evidence that commensal bacteria in the gut of animals acts as resistance gene reservoirs. MRSA strains with the ability to colonize and cause infections in humans have been documented in swine, cattle, and poultry (1, 13). It is also possible that bacteria harboring resistance genes are migrating from farms and sewage plants directly into natural environments, like streams and soil (14).

Previous research has indicated the success of quantification and isolation of bacteriophage DNA containing components of the SCC\textit{mec} element from the aforementioned environments (1, 15). However, there is currently no standard, optimized protocol for the handling and isolation of bacteriophage DNA from natural samples. As a means of increasing the likelihood of obtaining viral fractions with genes conferring antibiotic resistance, various procedures were performed to determine the optimal conditions for obtaining components of the SCC\textit{mec} element. Liquid and solid (mostly fecal matter and soil) environmental samples were obtained from areas frequented by
both domestic and wild animals due to the increased likelihood of obtaining viral fractions with resistance genes. Samples were continuously collected, and an optimized protocol was developed with the aim of detecting components of the SCC\textit{mec} element via PCR. Experimental variations during the procedure were implemented with the goals of minimizing the complications with processing environmental samples, such as DNA degradation and PCR inhibition.

**Methods**

**Sample Collection:** Solid samples were manually collected by researchers by shoveling matter into a plastic bag or small container, and stored at room temperature to be immediately processed. Liquid samples were manually collected by researchers from rivers and streams in bottles and refrigerated at 37°C until processing. Sewage samples were obtained manually in five-gallon containers with assistance from local sewage authorities and refrigerated at 37°C to be immediately processed. Samples that were obtained from external sources were shipped to James Madison University and stored at room temperature until processing. The majority of samples were collected from the Shenandoah Valley region of Virginia, including Harrisonburg, Staunton, Mt. Crawford, Elkton, and Basey, as well as the King George region of Virginia.

**Processing of Environmental Samples:** For the extraction of phages from a solid sample, samples were broken up and manually cleared of bulky environmental debris. Phosphate Buffered Saline (PBS) buffer containing 10.0 mL/L of 0.1 M MgSO$_4$, 10.0 mL/L of 0.4 M CaCl$_2$, 20.0 mL/L of 2.5 M Tris (pH 7.8), 5.9 g/L NaCl and 1.0g/L gelatin was prepared and added to the solid sample in a 2:1 buffer to sample ratio, and set to agitate overnight at 37°C. Extra solid debris was removed utilizing a cheesecloth filter as necessary. Samples were then centrifuged at 8,000 rpm for 40 minutes at 4°C to precipitate solid waste. Solid sample supernatant and unprocessed liquid samples
underwent gravity filtration with 2.5 μM pore sized Whatman® (GE Healthcare Bio-Sciences, Pittsburg, Pennsylvania) filter paper. Those samples with heavy particulate matter underwent gravity filtration with a consecutive pore-size gradient of Whatman® filter paper with approximately 0.8-0.4 μM pores, with intermittent centrifugation at the above specifications, until the buffered sample was moderately transparent. Centrifuged and gravity filtered sample underwent subsequent vacuum filtration with a ~0.22 μM Nalgene Thermo Scientific® (Thermo Fisher Scientific, Waltham, Massachusetts) filter to obtain a sterile sample and was maintained under sterile conditions for subsequent processing steps.

**Obtaining a Viral Fraction:** To obtain a viral fraction, a phage precipitant mixture was created. For every 200 mL of sterile, PBS-buffer-sample mixture, 5.8 g of NaCl (0.5 M) and 20g of Polyethylene glycol-PEG-8000 (10%) was added and shaken to dissolve salts and stored overnight at 4°C. Sample sizes ranged from 200 uL to 5 L. Samples were then centrifuged at 8,000 rpm for 45 minutes at 4°C to obtain a pelleted viral fraction. Pellets were resuspended in 1-2 mL of sterile water based on sample size, and vortexed briefly to homogenize.

**Sample Treatment Modifications:** Samples selected for further treatment underwent dialysis using a 1-3 mL Slide-A-Lyzer® Dialysis Cassette (3,500 MWCO) (Thermo Fisher Scientific, Waltham, Massachusetts) in ddH₂O at a minimum 1:3000 sample to ddH₂O ratio. Dialyzing samples were set on a stir plate at 37°C for minimum of 2 hours and a maximum of approximately 10 hours overnight with one ddH₂O change.

Selected samples were further treated with a Dnase and Proteinase K cocktail to eliminate DNA and protein contamination. Larger sterile samples were divided into microcentrifuge tubes to match the size specifications for future incubation. Proteinase K (final concentration 100 ug/mL, 1:200 Proteinase K to sample dilution) and 10x Dnase (1:1000 Dnase to sample dilution) were added to
each samples. Samples were inverted to mix and briefly centrifuged to remove contents adhering to the tube cap. All samples were then incubated for 30 minutes in a Fisher Scientific® dry bath incubator at 37°C, and heat shocked in a separate incubator at 65°C for 10 minutes to inactivate the enzymes.

**DNA Extraction, Quantification and Visualization:** To isolate phage genomic DNA, a Promega® Wizard DNA Purification system (Promega Corp., Madison, Wisconsin) was utilized. Sterile, processed or treated samples were transferred to sterile 14 mL tubes for increased space allocation. In the 14 mL tube, 2 mL of pre-warmed (37°C) Promega DNA-Clean Up Resin® (Promega Corp., Madison, Wisconsin) was added and mixed by gentle pipetting up and down to uncoat the phage particles. Next, 0.8 mL of water-resin-phage DNA solution was added to two Wizard® minicolumns and spun in a small, tabletop centrifuge for 1-2 minutes. The bottom column liquid was removed, and the remaining DNA solution from the sample was added to the top. The procedure was repeated until the entirety of each sample was used. Salts and proteins were washed off with 500 uL of 80% isopropanol in each column. The columns were spun in a small tabletop centrifuge for two minutes, and rotated 180 degrees to ensure removal of all salts and proteins, and then re-spun. To elute the phage genomic DNA, the column was transferred to a clean, autoclaved, labeled, microcentrifuge tube. Fifty uL of pre-heated 80°C de-ionized H2O was rapidly applied to the resin column. The mixture was left to sit for 30-60 seconds to release the DNA. Then, the mixture was spun at 13,000 rpm, at 25°C for 1 minute. Waste contents were removed and the process was repeated for the desired number of elutions. Samples that were processed with the modified Dnase and Proteinase K treatment were eluted in 80°C TE buffer in lieu of diH2O. Extracted DNA elutions were quantified via NanoDrop Spectroscopy (Thermo Scientific Corp., Wilmington, Delaware) in ug/mL. Nanodrop readings were performed until elutions indicated DNA concentrations within 10-20 ug/L. Approximately 30 ng of selected DNA samples were analyzed
with 1% agarose gel electrophoresis for visualization of potential DNA degradation. DNA samples with viable DNA were run on Low-Melt Agarose gel electrophoresis for band excision and excised band PCR.

**DNA Amplification:** A Qiagen® Toptaq Mastermix Kit (Qiagen® Inc., Valencia, California) containing a 2x TopTaq mastermix, TopTaq DNA Polymerase and 10x CoralLoad® Concentrate was utilized for PCR reactions. CoralLoad® Concentrate PCR Buffer contains gel-tracking dyes enabling immediate gel-electrophoresis loading of PCR products (Table 1a). Reaction components were standardized for 20 uL reactions and adjusted as needed for desired variation in reaction sizes and measured sample DNA concentrations (Table 1b).

**Table 1a.** Toptaq Mastermix components utilized for PCR amplification. Protocol and components provided by Qiagen® (Qiagen Inc., Valencia, California).

<table>
<thead>
<tr>
<th>Mastermix Component</th>
<th>Volume/Reaction</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x TopTaq PCR Buffer</td>
<td>5uL</td>
<td>1x</td>
</tr>
<tr>
<td>dNTP mix (10mM)</td>
<td>1uL</td>
<td>200 uM of each dNTP</td>
</tr>
<tr>
<td>10x CoralLoad Concentrate</td>
<td>5uL</td>
<td>1x</td>
</tr>
<tr>
<td>Toptaq DNA polymerase</td>
<td>0.25uL</td>
<td>1.25 units/reaction</td>
</tr>
</tbody>
</table>

**Table 1b.** Multiplex PCR reaction components utilized for samples and controls.

<table>
<thead>
<tr>
<th>Multiplex PCR Reaction: Sample Mixture</th>
<th>Multiplex PCR Reaction: Control Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x TopTaq® Mastermix- 10uL</td>
<td>TopTaq® Mastermix- 10uL</td>
</tr>
<tr>
<td>Sample DNA- 9uL</td>
<td>Control DNA- 1uL</td>
</tr>
<tr>
<td>Multiplex Primer: 1uL</td>
<td>RNase Free H₂O- 9uL</td>
</tr>
</tbody>
</table>
**Multiplex Primer System:** Multiplex primers for both the *mec* and *ccr* gene regions were created according to the methods of Kondo *et al.* 2009 (Tables2a/b). The optimal annealing temperature for primers mA1/mA2 and mecC_fwd/ mecC_rev was determined via a PCR temperature-gradient at four temperatures (54.0°C, 55.1°C, 57.6°C) (Brooke Sauder, personal communication). Multiplex PCR amplification was performed on a My Cycler® Thermalcycler (Bio-Rad Laboratories Inc., Hercules, California) utilizing the following steps: initial denaturation at 94°C for 2 minutes, followed by 94°C for 2 minutes; annealing at 57.6°C for 1 minute; extension at 72°C for 2 minutes, and cycling 30 times. This was followed by an extension at 72°C for 10 minutes. MecA and ccr controls are listed in Table 3.

**Table 2a.** *mec* multiplex primers, gene regions detected based on primer pair, and product size for different SCCmec elements.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Gene or region detected (primer pair)</th>
<th>Nucleotide sequence 5'-3'</th>
<th>Expected product size</th>
<th>SCCmec Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>mA1</td>
<td><em>mecA</em> (mA1/mA2)</td>
<td>TGCTATCCACCCTCAAACAGG</td>
<td>286bp</td>
<td>II</td>
</tr>
<tr>
<td>mA2</td>
<td><em>mecA</em> (mA1/mA2)</td>
<td>AACGTTGTAAACCACCCCAAGA</td>
<td>286bp</td>
<td>II</td>
</tr>
<tr>
<td>mecC_fwd</td>
<td><em>mecC</em> (mecC500_fwd/mecC500_rev)</td>
<td>GCCGTGTTTATCCATTGAACGAAA</td>
<td>496bp</td>
<td>XI</td>
</tr>
<tr>
<td>mecC_rev</td>
<td><em>mecC</em> (mecC500_fwd/mecC500_rev)</td>
<td>TGGGTTGAAACGTTGTAGG</td>
<td>496bp</td>
<td>XI</td>
</tr>
</tbody>
</table>
Table 2b. Ccr multiplex primers, gene regions detected based on primer pair, and product size for various SCC \textit{mec} elements.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Gene or region detected (primer pair)</th>
<th>Nucleotide sequence 5’-3’</th>
<th>Expected product size</th>
<th>SCC \textit{mec} Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1</td>
<td>ccrA1 (α1-BC)</td>
<td>AACCTATATCATCAATCAGTACGT</td>
<td>695</td>
<td>I</td>
</tr>
<tr>
<td>α2</td>
<td>ccrA2 (α2/bc)</td>
<td>TAAAGGGCATCAATGCACAAACT</td>
<td>937bp</td>
<td>II</td>
</tr>
<tr>
<td>α3</td>
<td>ccrA3 (α3/bc)</td>
<td>AGCTCAAAAAGCAAGCAATGAAT</td>
<td>1791bp</td>
<td>III</td>
</tr>
<tr>
<td>βC</td>
<td>ccrB1, ccrB2, ccrB3</td>
<td>ATTCGGTTGATAATAGCCITCT</td>
<td>---</td>
<td>I-III</td>
</tr>
<tr>
<td>α4.2</td>
<td>ccrA4 (α4.2/β4.2)</td>
<td>GTATCAATGCACCAGAACTT</td>
<td>1287bp</td>
<td>VI</td>
</tr>
<tr>
<td>β4.2</td>
<td>ccrB 4.2</td>
<td>TTGCGACTCTCTTGGCGTTT</td>
<td>1287 bp</td>
<td>VI</td>
</tr>
<tr>
<td>yR</td>
<td>ccrC (yR-yF)</td>
<td>CTTTATAGACTGGATTATTCAAATAT</td>
<td>518bp</td>
<td>V</td>
</tr>
<tr>
<td>yF</td>
<td>ccrC (yR/yF)</td>
<td>CGTCTATTACAAGATGTAAGATAAT</td>
<td>518bp</td>
<td>V</td>
</tr>
</tbody>
</table>
Table 3. MecA and ccr multiplex controls and corresponding SCCmec type. *S. aureus* strain RN4220, cloning intermediate, was utilized as a negative control for both multiplex sets.

<table>
<thead>
<tr>
<th>mecA Multiplex Controls</th>
<th>ccr Multiplex Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive: SCCmec COL (Type I)</td>
<td>Positive: SCCmec N315 (Type II) or SCCmec COL (Type I)</td>
</tr>
<tr>
<td>Negative: RN4220</td>
<td>Negative RN4220</td>
</tr>
</tbody>
</table>

Inhibition PCR Tests: The potential for PCR inhibition was simultaneously tested for selected samples via a designed PCR inhibition test. Sample DNA was combined with multiplex-specific positive control DNA, and underwent amplification under the previously stated conditions (Table 4). Sample DNA volume varied for a total concentration of approximately 30 ng/uL. RNase-Free H₂O was utilized to eliminate potential RNase contamination, adjusted as needed for a 20 uL reaction.

Table 4. Inhibition testing reaction mixture components.

<table>
<thead>
<tr>
<th>Inhibition Testing: Reaction Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>10uL TopTaq® Mastermix</td>
</tr>
<tr>
<td>1uL Multiplex Mastermix Primer</td>
</tr>
<tr>
<td>(Approximately 30ng/uL) Sample DNA</td>
</tr>
<tr>
<td>Varied : RNase Free H₂O</td>
</tr>
<tr>
<td><strong>Total: 20uL</strong></td>
</tr>
</tbody>
</table>
**Gel Electrophoresis:** Processed and amplified samples were run on 1.5% SeaKem® Agarose gel (Lonza Bioscience, Walkersville, Maryland) containing 0.48 g agarose, 40 mL 1X TAE buffer and 1 uL ethidium bromide. Loaded samples were run at 150 V for approximately 40 minutes. Inhibition tests were comparatively analyzed next to their multiplex amplified, sample-only reaction to visualize potential PCR inhibition.

**Results**

**Processing of Environmental Samples:** Approximately 74 environmental samples were processed from 2012 to April 2014, from over 30 different geographic locations in Virginia and various U.S locations, including horse, cow, and poultry farms, local sewage authorities, lakes and streams, local parks and wooded areas (Figure 1). Solid and liquid samples were manually collected and were obtained in mass quantities to be consecutively processed from a sample stock in approximately 300-500g increments, and from 200mL-5L increments based on sample type and space availability.
Figure 1. (a) Percentage of environmental sample type for approximately 74 samples. (b) Geographic location of samples processed. Locations within the Shenandoah Valley, Virginia include the following listed by majority: Harrisonburg, Staunton, Mt. Crawford, Elkton, and Basey. Northern Virginia Samples were obtained from the King George region.

Of the 74 total documented and processed samples, approximately 40, mostly soil samples were processed utilizing a protocol developed by James Madison University researchers. This initial protocol was designed to extract potential phage DNA from environmental samples and detect the presence of the SCCmec element, by surveying previous environmental bacteriophage isolation procedures as performed by Colomer et. al 2011. An initial protocol, designated as “Protocol 1,” involving various filtration, purification, extraction, quantification and analysis steps was generated (Figure 2) and performed as described in the methods. The protocol was designed to first maximize exposure of the surface area of solid samples; samples were then placed in a Staphylococcus bacteriophage-specific PBS buffer on a stir plate at optimal temperature and conditions (37°C, pH 6-7) to homogenize and support bacteriophage propagation (16). Centrifugation was utilized to eliminate pelleted environmental debris, while retaining a phage-containing supernatant, and gravity filtration was utilized to eliminate coarse environmental materials, followed by subsequent vacuum filtration to eliminate potential microbial contaminants.
Figure 2. Protocol 1. Initial protocol used for isolation and detection of *S. aureus* bacteriophage antibiotic resistance genes.

**Multiplex PCR positive results:** Isolated DNA was quantified and subject to *mecA* and *ccr* multiplex PCR and gel electrophoresis. Isolation of *mecA* and *ccr* positive products was successfully performed by Brooke Sauder (Figure 3). One sample was successfully transformed into *E. coli* using a TOPO® TA (Life Technologies™, Carlsbad, California) cloning vector, and sequenced revealing that it was SCC*mec* type I.2 (not listed).
Figure 3. *Mec* and *ccr* Multiplex positive samples processed using “Protocol 1”. PCR amplification and Gel Electrophoresis was performed by Brooke Sauder. Gel key lists sample titles, followed by the predicted gene amplified, gene product size, and the predicted primer pair apart of the corresponding multiplex.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample Name</th>
<th>Predicted Gene</th>
<th>Approximate Size</th>
<th>Predicted Primer Pair</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:</td>
<td>NTM Compost</td>
<td><em>mecA</em></td>
<td>500 bp</td>
<td><em>mecC500_fwd/rev</em></td>
</tr>
<tr>
<td>2:</td>
<td>NTM Gold Compost</td>
<td><em>ccrA1-ccrB</em></td>
<td>600 bp</td>
<td><em>a1/bC</em></td>
</tr>
<tr>
<td>3:</td>
<td>NTM Gold Compost</td>
<td><em>mecA</em></td>
<td>500 bp</td>
<td><em>mecC500_fwd/rev</em></td>
</tr>
<tr>
<td>4:</td>
<td>1 Kb Ladder</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5:</td>
<td>100 bp Ladder</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6:</td>
<td>NTM barn</td>
<td><em>ccrC</em></td>
<td>500 bp</td>
<td><em>yR/yF</em></td>
</tr>
<tr>
<td>7:</td>
<td>NTM barn</td>
<td><em>mecA</em></td>
<td>500 bp</td>
<td><em>mecC500_fwd/rev</em></td>
</tr>
<tr>
<td>8:</td>
<td>C-Sterile 1</td>
<td><em>ccrA1-ccrB</em></td>
<td>600 bp</td>
<td><em>a1/bC</em></td>
</tr>
<tr>
<td>9:</td>
<td>C-Sterile 2</td>
<td><em>ccrA1-ccrB</em></td>
<td>600 bp</td>
<td><em>a1/bC</em></td>
</tr>
</tbody>
</table>

PCR positive results and subsequent cloning confirmed the viability of the basic protocol in isolating and detecting the *mecA/ccr* gene targets, however positive results were highly infrequent or extremely difficult to repeat or obtain, despite assertions of the ubiquity of the gene targets in environmental samples in a previous study conducted in Spain (1). Of the samples processed utilizing the initial protocol, Nanodrop spectroscopy indicated consistently low DNA yields for all sample types (Figure 4). DNA degradation was also evident by gel electrophoresis (not shown).
Figure 4. DNA yields for approximately 40 samples processed utilizing the designed protocol, Protocol 1.

Due to low DNA yields, evident DNA degradation, and consistently negative PCR samples, the potential for environmental factors present in the sample aiding in DNA degradation and PCR inhibition was explored. “DNA inhibition tests” were designed and performed as described in the methods by adding positive control DNA to a sample PCR reaction before undergoing amplification in addition to the standard multiplex PCR procedure. Inhibition testing in conjunction with multiplex PCR allows for either the elimination or confirmation of the presence of PCR inhibitors, as well as the confirmation of negative, yet successfully amplified, PCR products. Inhibition testing of the degraded, isolated DNA indicated PCR inhibition was present in “Goat Sample” (Figure 5).
Figure 5. DNA inhibition test indicating the presence of PCR Inhibitors for DNA isolated from the indicated samples using “Protocol 1”. Testing was performed using the ccr Multiplex primers and COL strain DNA as a positive control, with positive bands at 697 bp. Sample DNA from “Goat Sample” (103.6 ng/μL) and “Indian Lake” (44.7 ng/μL) were combined with COL DNA prior to amplification and analyzed for inhibitors. Lanes contain the following: Lane 1, COL positive control; Lane 2, COL and Goat Sample; Lane 3, COL and Indian Lake Sample; Lane 4, 1Kb Ladder (New England Biolabs Inc.®, Ipswich, Massachusetts).

Protocol Modifications: Stepwise modifications and variations to Protocol 1 were performed over the course of 2012 to 2013 with the goal of establishing optimal conditions for the isolation of *S. aureus* bacteriophages, while eliminating PCR inhibitors, and minimizing DNA loss or degradation. In terms of sample collection, new emphasis was placed on samples isolated from farms and sewage rather than soil samples, due to the previously indicated presence of *S. aureus* bacteriophages in these locations. Further emphasis was placed on obtaining and processing mass quantities of sample to increase the probability of obtaining and amplifying the target DNA (11). Due to the increasingly murky nature of the processed sewage and fecal samples, routine centrifugation following by a pore-sized gradient gravity filtration was utilized to effectively eliminate solid matter for subsequent vacuum filtration. Viral fraction precipitation was performed as previously stated, however subsequent dialysis and DNase and Proteinase K treatments were added as crucial modifications for
sample purification. Dialysis was utilized to facilitate the removal of buffer salts, small protein contaminants, and macromolecules present in the sample that are larger than 3500 Daltons. DNase treatment was utilized to remove extra-phage capsid DNA particles that may be present in the sample. Further, *Staphylococcus aureus* produces heat stable nucleases, resistant to denaturation, which may be present in the phage precipitate and degrade the phage DNA once it is released from the phage capsid. Therefore, Proteinase K was added to degrade present nucleases and extra protein contamination (17). DNA visualization via gel electrophoresis was utilized to test for degradation prior to multiplex PCR amplification. Inhibition testing as previously described was utilized to further test for inhibitors and confirm successful PCR amplification and sample negativity. This new protocol was generated and designated as Protocol 2 (Figure 6).
Nanodrop spectroscopy did not indicate significantly higher DNA yields, despite larger sample sizes processed and prior treatments. However, DNA degradation was not evident in samples after DNA gel electrophoresis (Figure 7). “VCU Waste” sample DNA was not visible, however “Richmond” Sample DNA was visible and indicated a lack of degradation.

Figure 7. Isolated DNA utilizing Protocol 2. Sewage sample DNA from Richmond, Virginia titled VCU Waste sample (9.9 ng/uL) and Richmond Sample (19 ng/uL) were compared to positive control strain N315 DNA. Lane contain the following: Lane 1, 1 Kb Ladder; Lane 2, VCU Waste Sample DNA; Lane 3, Richmond Sample DNA; Lane 4, N315 positive control DNA; Lane 5, No DNA, negative control.

DNA inhibition testing experiments as described in the methods were simultaneously run as a standard in the modified protocol to control for the potential for DNA degradation as pictured below (Figure 8). Gel electrophoresis indicated a lack of PCR inhibition.
Figure 8. DNA inhibition test for DNA isolated from the indicated samples using Protocol 2. Testing was performed using both the mec and ccr multiplex primers and the N315 strain DNA as a positive control, with positive bands at 400 and 500bp for the mec multiplex and 697 bp for the ccr multiplex, indicated by arrows. Gel key indicates multiplex type/sample name and presence of positive control DNA. Sample DNA from Compost 1 (10.7 ng/µL) Compost 2 (41.1 ng/µL) and VCU 2 samples were combined with COL DNA prior to amplification and analyzed for inhibitors. The bands near the lower set of wells represent unused primers and primer dimers.

Certain samples were processed, utilizing both Protocol 1 and Protocol 2 for the same original sample, to test for potential differences in inhibition between the two samples. Variation in inhibition between protocols was not evident, as all were successfully PCR amplified as indicated by inhibition testing (not shown). Selected sample DNA isolated utilizing Protocol 1 was also subject to gel electrophoresis and excised to undergo PCR amplification as an alternative method of
puriﬁcation. All samples were subject to multiplex PCR as previously described. Positive multiplex PCR products were not detected despite viable DNA samples (Figure 9).

![Image](image1.png)

**Figure 9.** *Mec* and ccr multiplex PCR ampliﬁcation performed on Richmond and VCU Sewage samples with various treatments. DNA extraction using protocol 1: Richmond and VCU samples. DNA extraction using Protocol 2: Richmond Treated and VCU Treated samples. Richmond Excised DNA was excised gel DNA isolated using Protocol 1. (A) *mec* multiplex PCR products. Lanes contain the following: Lane 1: 1 Kb ladder (New England Biolabs Inc.®, Ipswich, Massachusetts); Lane 2: Richmond sample; Lane 3: Richmond Treated sample; Lane 4: Richmond Excised sample; Lane 5: VCU; Lane 6: VCU Treated sample; Lane 7: N315 positive control (400 and 500 bp); Lane 8: H₂O negative control. (B) ccr multiplex PCR products. Lanes contain the following: Lane 1: 1 Kb ladder (New England Biolabs Inc.®, Ipswich, Massachusetts); Lane 2: N315 positive control (697 bp); Lane 3: Richmond; Lane 4: H₂O negative control.

**Overall Summary:** Of the over 70 processed samples, multiplex PCR results indicate 27% PCR positive results for components of the SCC*mec* element for both protocols. Of the positive results, ccr gene products detected were slightly higher than *mec* gene products (Figure 10).
**Figure 10.** Overall summary *mec* and *ccr* multiplex positives for over 70 samples processed from 2012 to April 2014 using both Protocol 1 and Protocol 2.
Discussion

The development of an optimized protocol for isolation and detection of regions of the SCCmec element in phage DNA from environmental samples was developed over the course of two years in an effort to provide reliable and reproducible results for further and ongoing research. Of the over 70 processed samples, the mecA gene was detected in 22% of samples and the ccr gene was detected in 33% of all samples (Figure 10). Previous literature has indicated successes in the detection of the mecA gene in 80-100% of samples with a lack of detailed methods outlining the isolation of phage DNA and a sound confirmation of positives (1, 15). Researchers from James Madison University began in 2012 to process various samples from similar agricultural locations in an effort to reproduce these findings. Colomer et. al (2011) reported quantification of mecA via qPCR of viral fractions of 71 samples, with approximately 60 samples containing detection of the mecA gene, and approximately 40 containing over two log10 of gene copies/ml of isolated mecA DNA detected per sample, with a significantly higher (p<0.05) prevalence in swine and poultry farm soil than fecal samples (1). However, no agarose gel analysis of the products was shown, nor were any genes confirmed by sequencing. Contrary to these results, of the over 74 samples processed in mass quantities in this study, 15 samples indicated the presence of any of the mecA subclasses, one of which was sequenced and confirmed positive. While ccr gene detection was slightly higher than mec gene detection in this study (Figure 10), a complete mec complex containing the mecA gene and regulatory genes is believed to be the necessary requirement for the expression of methicillin resistance. Therefore, the initial focus of this study was similarly on detecting the mecA gene, as the mecA gene has never been observed to be transferred independently of its regulatory genes. Further, due to the large size of the SCCmec element (between 21 and 67 Kb), it is more likely that a piecewise gene transfer via bacteriophage transduction occurs, which may lead to differences in mec and ccr gene detection (1). It has also been suggested that the smaller SCCmec classes, from IV and
onwards are more easily transferred between strains, as they are less of a fitness burden to the bacterial cell (18). Despite Colomer et. al's (2011) inferences of the natural abundance of the SCC\textit{mec} element in the environment based on the large number of \textit{mecA} gene copies they detected (1, 15), it is important to note that the acquisition events of the element itself are still somewhat speculative and currently hypothesized to be somewhat restricted in \textit{S. aureus} strains compared to antibiotic resistance acquisition in related strains, such as \textit{S. epidermidis}. It is believed that not all \textit{S. aureus} genetic backgrounds are conducive to maintaining an active, plasmid-derived \textit{mecA} gene, a phenomenon titled “the barrier effect” which could potentially contribute to the low number of MRSA lineages identified in epidemiological studies (19). In Katayama et. al (2003) \textit{mecA} was observed to be highly influenced by chromosomal elements outside of SCC\textit{mec}, alluding to the possibility of certain strains as able or unable to “host” \textit{mecA} based on their genetic make-up (20).

Therefore, acquisition of the SCC\textit{mec} element via phage transduction may not be as prevalent in \textit{S. aureus} strains present in the environment as Colomer et. al's (2011) results would suggest, and could explain the observed variation in this study due to an inability to acquire the element. However, an inability of \textit{S. aureus} strains to acquire the element does not fully explain the general lack of detection in this study’s environmental samples. Further testing aiming to transduce these genes experimentally could be performed to elucidate and confirm these mechanisms; Colomer et. al (2011) highlights the value of such experiments in an effort to confirm their assertions, yet experimentation proved extremely difficult and unsuccessful due to the difficulty of replicating optimal conditions for transduction to occur (15).

In another study by Colomer et. al in 2011, 15 river water and urban sewage samples from Barcelona, Spain were processed and the presence of \textit{mecA} in both bacterial and phage DNA was detected in each sample, with a greater number of overall bacteriophage gene copies (in log_{10} gene copies/mL) in river water samples as opposed to sewage (15). The higher prevalence of \textit{mecA} in river
water phage fractions was further hypothesized to result from the presence of both human and animal fecal pollution, in addition to bacteria present in river water, however, since the copy number of resistance genes per cell is unknown, gene copy abundance does not necessarily refer to the number of original carrier bacteria or phages present (21). These results indicated unprecedentedly high levels of mecA in municipal wastewater samples while the study omitting pertinent DNA sequencing information that confirmed their findings.

In an effort to replicate the high success of isolation from these environments, the methods in these studies were analyzed and performed based on the listed protocol. Modifications were implemented as an effort to eliminate the unexpected difficulties that were encountered over the course of this study, which were highly variable in comparison to the literature. Of notable importance in variation between both studies was the presence of PCR inhibitors and impurities present in the sample, which could contribute to the degradation of the target DNA. DNA extraction procedures may be insufficient in removing PCR reaction inhibitors such as detergents or surfactants, humic substances, and aromatic compounds present in sewage samples. Such organic and inorganic compounds can inhibit Taq polymerase by binding to nucleotides and magnesium ions, rendering them unavailable to the polymerase, particularly in qPCR applications (21).

The indicated presence of PCR Inhibitors in this study (Figure 5) brings into question the inherent differences in environments in which the samples were obtained; it is possible to argue that water and soil samples from Barcelona, Spain naturally contain different or less potential inhibitors and more antibiotic resistance genes. Factors such as upstream population density, proximity of wastewater treatment plants and animal feeding plants have all been shown to directly correlate with prevalence and detection of antibiotic resistance genes (22). However such high detection of mecA bacteriophage density in municipal water samples has not been reported in other locations (21).
Previous experimentation has indicated that the quality of the extracted DNA is crucial to the applicability of PCR and the detection of gene targets in complex aquatic environments (21), yet this difficulty was not reported by Colomer et. al (2011). The initial focus at the start of our protocol development on processing mass quantities of sample for increased DNA yield was diverted to maintaining DNA purification based on evident DNA degradation and a lack of correlation between sample size and DNA detection by spectroscopy. In a previous method development study by Burgmann et. al (2001), optimization of the quality and quantity of DNA extracted from soil was explored from samples representing a range of typical European soils, with respect to pH, texture and organic matter content. Results indicated unequal efficiency in DNA extraction, DNA fragment sizes, and levels of PCR inhibition across soil samples, which are hypothesized as resulting from the complex variations in densities, particle size distributions, and water contents of very different soils. Interestingly, all soil samples contained a range of PCR sensitivity to total PCR inhibition, which was shown to increase with smaller sample sizes (23). Such obstacles may not be entirely possible to completely overcome in a standardized protocol, but may be minimized using extensive purification methods.

The more rapid degradation of bare mobile genetic elements may also enhance the possibility of DNA degradation; however, mobile genetic elements are protected from factors such as temperature and nuclease sensitivity when present in a phage capsid. Therefore, the treatments in Protocol 2 were utilized to eliminate the possibility of environmental contaminants present in the sample prior to breaking of the phage capsid during the DNA extraction step. The methods presented in the Protocol 2 define a concise outline for the purification of bacteriophage DNA specifically for highly contaminated, environmental samples, which has remained relatively unaddressed. Both the above protocol and Colomer et. al’s (2011) protocol contain pore filtration and centrifugation before a sterilization step; however in addition, Protocol 2 includes a membrane
based dialysis step for every processed sample followed by a proteinase K and DNase cocktail prior to DNA extraction. Both protocols utilize DNase treatment prior to DNA extraction to degrade extra-phage DNA, and in addition, an optional control of non-phage DNA may be utilized to rule out the presence of bacterial or non-encapsidated DNA, after DNase treatment but prior to desencapsidation. DNA extraction procedures were kept unmodified in Protocol 2 due to the initial detection of both mec and cer genes (Figure 3). The multiplex PCR system employed in the above protocols was utilized to both potentially amplify multiple SCCmec gene components, as well as to identify the SCCmec type. Identifying the SCCmec type is believed to be useful in defining MRSA clones for epidemiological studies, which are increasingly divergent in nature (9). Gel electrophoresis identification of potential PCR products in conjunction with inhibition tests provides a means of visualizing target genes or confirming that a sample DNA does not contain the target genes, if indicated as uninhibited and successfully amplified (Figure 8). Due to the complexity of the processed samples, as well as the frequent presence of inhibitors, the above inhibition tests were implemented as a standard procedure rather than a mechanism for troubleshooting. As previously stated, Colomer et. al (2011) has reported, in one case, a 100% positive sample rate without gel electrophoresis results, or DNA sequencing information, which are invaluable to the reliability of these results, especially due to the possibility of bacterial DNA cross contamination.

Ultimately, the reliability of any method that seeks to isolate a target gene must be enhanced by sequence-based typing methods. Sequencing typing of mobile genetic elements like SCCmec combined with the thousands of typed MRSA strains are used to gain insight into the evolution of both MRSA strains and even the SCCmec element (24). A currently unresolved question remains whether MRSA outbreaks originate from the proliferation of individual, resistant clones, or rather by independent acquisition of SCCmec elements by methicillin-susceptible S. aureus (MSSA) strains. Further DNA analysis has interestingly revealed that the most prevalent MRSA clones associated
with hospital acquired infections toady were prevalent amongst MSSA strains 40 years ago, in various European countries, which may support the belief of independent acquisition of the SCC\textit{mec} element (25). Presently, the identification of SCC\textit{mec} elements by sequencing may give the most insight into the complex evolutionary mechanisms and virulence associated with MRSA.

Overall, the purpose of this study was to generate a protocol for isolation and detection of bacteriophage transfer of the SCC\textit{mec} element in environmental samples, due to the inconsistencies associated with replicating previously reported results. Overall, 22% of the processed samples indicated presence of the mec\textit{A} gene in viral fractions. The implementation of purification treatments and inhibition testing as outlined in Protocol 2 has proven crucial to combating the difficulties associated with processing complex environmental samples. The experimentation that resulted in these modifications was performed with the same ultimate goal as the research that inspired this study; to detect environmental reservoirs of antibiotic resistance in order to elucidate the mechanism by which bacteria evolve resistance to antibiotics. Confirming how virus are able to transfer resistance will be the start for developing new medicines and phage therapies that will take a new approach to circumvent the mounting antibiotic resistant bacteria proliferating in communities and hospitals.
Bibliography


