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Identification of the Staphylococcal Cassette Chromosome Mec (SCCmec) element in viral fractions from environmental samples

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Identification of the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) Element in Viral Fractions from Environmental Samples

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Abstract

Antibiotic resistance attributed to methicillin-resistant *Staphylococcus aureus* (MRSA) has become a growing concern over the last decade in both the healthcare and agricultural environment. This resistance is encoded by the gene *mecA* that is located on a mosaic, mobile genetic element called the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) element. It is proposed that the transfer of the SCC*mec* element and resulting spread of resistance occur by transduction, the transfer of genetic material from bacterium to bacterium by a bacteriophage. Specifically, it is hypothesized that the transduction of this resistance is occurring in the agricultural setting. To test this, a protocol was optimized to allow for an efficient filtration of the environmental samples and a high yield of concentrated viral DNA. It was determined that 22% of the total samples collected contained either the *mec* gene or the *ccr* gene, while 9% of the total samples contained both antibiotic resistance genes. While it was determined that the protocol did not affect the generation of a PCR product, inhibition PCR manifested the presence of inhibitors in different samples, which may have contributed to a "negative" PCR product. These findings manifested the presence of methicillin resistance in environmental samples from local areas in Virginia. These results have direct implications on antibiotic use in agriculture and should be a cause for alarm.

I. Introduction

Antibiotic resistance has become an important, growing concern over the last decade. Specifically, methicillin-resistant *Staphylococcus aureus*, commonly known as MRSA, has been a large focus of this concern. This antibiotic-resistant strain of *S. aureus* is a gram positive bacterium typically found on the skin or in the nose. Once the skin or mucosal barriers have been breached, MRSA can spread and cause illness ranging from a minor skin infection to a life-threatening disease, such as pneumonia. Accounting for many hospital-acquired infections in the early 2000s (1), MRSA has also caused infection in the community, outside of the healthcare system. Additionally, the increased use of antibiotics in agriculture, for farming and livestock feeding purposes, combined with the natural occurrence of antibiotics in the soil have allowed for interest in the spread of MRSA in the environment.

Methicillin resistance is encoded by a gene called *mecA*. This gene codes for a mutation in a penicillin-binding protein PBP-2A (1). This mutation causes a lower affinity to bind to penicillin and thus penicillin resistance. The *mecA* protein also confers resistance to other beta-lactam antibiotics, such as oxacillin and flucloxacillin (1).

mecA is located on the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) element (Fig. 1). The SCC*mec* element also carries the *ccrAB* gene, which encodes the mobility of this genetic element. The *ccrAB* gene, composed of one or two site-specific recombinase genes, encodes the excision and integration of SCC*mec* element at specific integration sites, such as *attA* and *attB* (2). Currently, there are about eleven known different types of SCC*mec* based on the different combinations of classes and types of the *mecA* and *ccrAB* genes (3). However, because SCC*mec* is such a mosaic element, many more types are predicted to be discovered.

Figure 1. Basic components of the SCC*mec* element.

Because the general topic of antibiotic resistance has been a large focus in research in recent years, many studies have been published proposing mechanisms of transfer of resistance. Some hypothesize that the resistance is transferred between bacterial cells by the reorganization of the membrane and its permeability, decrease of porin content, and/or the over expression of the efflux pumps (4). These changes would not result from a genetic change. However, another proposed mechanism is that the genetic composition of the bacteria changes either by the acquisition of "mutator genes" (5) or by a method of horizontal gene transfer.

Presently, the mechanism of transfer of the SCC*mec* element, and thus the transfer of *mecA*, is unknown. While conjugation is a horizontal gene transfer method commonly known to transfer resistance genes on plasmids in *S. aureus* (6), many are unsure as to how the SCC*mec* element, a chromosomal portion of the DNA, is transferred. Transduction, another method of horizontal gene transfer, is proposed to be a possible mechanism of this transfer (Fig. 2). This method involves the use of bacteriophages, viruses that infect bacteria, as vectors in the transfer of bacterial DNA between different bacterium. It is possible that MRSA acquires the SCC*mec* element via bacteriophages in the environment, resulting in the spread of beta-lactam antibiotic resistance, such as resistance to oxacillin and flucloxacillin.

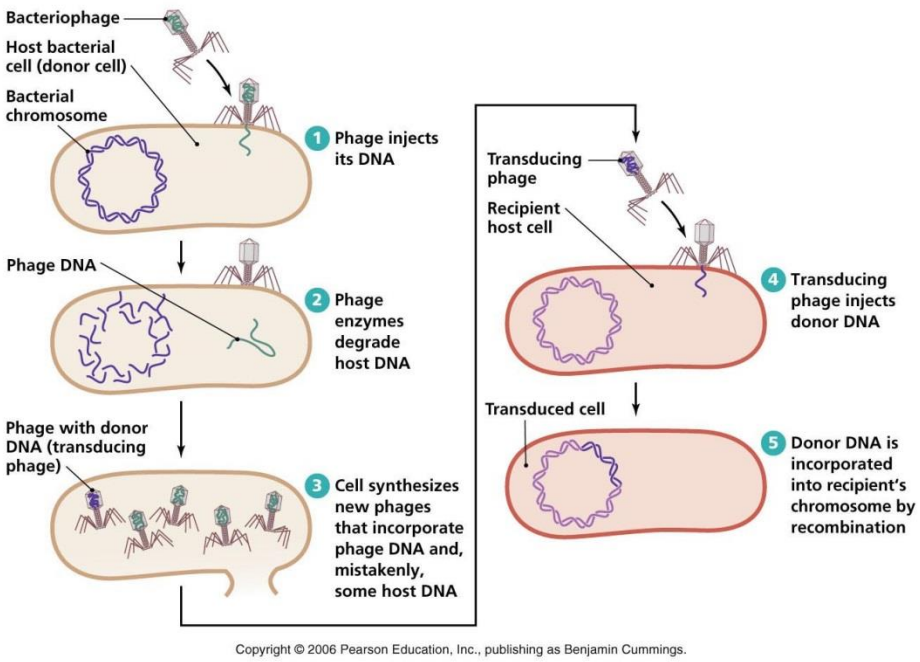

Figure 2. A schematic showing the proposed method of generalized transduction. While this is general, it can be applied to the transfer of the SCC*mec* element. After infecting a methicillin resistant *S. aureus* bacterium (host bacterial cell), the environmental bacteriophage repackages genetic material into its capsid, some of which may contain the SCC*mec* element. The same bacteriophage infects another *S. aureus* bacterium that is susceptible to methicillin and other beta lactam antibiotics (recipient host cell). If the SCC*mec* element is inserted into the bacterial chromosome, the bacterium becomes resistant to methicillin (transduced cell). Diagram taken from *Microbiology*, Pearson Education (2006), published by Benjamin Cummings.

Thus, it is possible that bacteriophages isolated from the environment, from fecal matter from farm animals, runoff water, and soil from an agricultural area, would have the SCC*mec* element in its genome. The genes *mecA* and *ccrAB* would be identified, indicating the spread of antibiotic resistance by transduction.

Scientists in Spain reported that they identified *mecA* and *blaZ*, another resistance gene, in an environmental bacteriophage population, from urban sewage and river water samples (7). In addition, they identified the genes in fecal waste samples form cattle, pigs, and poultry (8). However, these two publications did not provide the methodology used to isolate and identify these genes from the samples. Thus, a clear, efficient set of methods needed to be determined to

effectively isolate and identify SCC*mec* from any environmental samples collected. It was important to determine the most efficient filtration system to produce easily manipulated samples, the most efficient sterilization system to yield solely viral samples, and the most efficient DNA isolation system to produce a high yield of genetic information and amount of bacteriophage.

II. Methods

These methods consist of core methods and optimization methods. The core methods were sample collection, phage extraction without detergent, high centrifugation and filtration, phage precipitation with PEG and NaCl, DNAse treatment, DNA extraction using the DNA Clean-Up Resin kit, and multiplex PCR. The optimization methods were phage extraction with detergent, low centrifugation and filtration, phage precipitation with $Ca_3(PO_4)_2$ and Na_3PO_4 before PEG and NaCl treatments, dialysis, glycerol treatment, phenol/chloroform DNA extraction, and temperature gradient PCR.

Sample Collection

A large amount of environmental sample was collected. This sample was fecal matter from a farm animal, runoff water from a nearby farm, soil from an agricultural area, sewage, etc. A large amount of sample (about 1000 g or 1 L) was collected. Before the next step, the large sample was broken up into 100 g or 100 mL portions. If only a small amount of environmental sample was collected (less than 1000 g or 1 L), then the collected mass or volume of the sample was directly taken through the protocol.

In addition to samples analyzed with this protocol, a control phage lysate was created to observe if a specific step inhibited the phage. The control phage lysate was made by transducing 80α phage and *S. aureus* RN4220. That strain of *S. aureus* contained a plasmid pWA46 that contained the *ccr* gene on it. Once the lysate was created, it was added to either a volume of phage buffer or a volume of sample right before phage precipitation, which was termed "spiking" the sample.

Phage Extraction

For solid samples, the sample was first broken up into smaller pieces. Liquid samples were swirled to mix. Then, 10X phage buffer was added to the sample in a 2:1 mass: volume ratio and set to agitate overnight in a shaker at 4°C. [The 10X phage buffer recipe, for a total volume of 1.0 L, was: 10 mL of 1.0 M $MgSO_4$ (Thermo Fisher Scientific, Waltham, MA), 40 mL of 1.0M $CaCl_2$ (Thermo Fisher Scientific, Waltham, MA), 500 mL of 1.0 M Tris (Thermo Fisher Scientific, Waltham, MA) at 7.8 pH, 59 g of NaCl (Thermo Fisher Scientific, Waltham, MA), 10 g of gelatin (Thermo Fisher Scientific, Waltham, MA), and an amount of deionized water to achieve 1 L total.]

Detergent was additionally added to mixture of the sample and 10X PBS. The detergents used were 0.5% Tween20 or 1% Triton. Either detergent was added in those concentrations to the mixture before filtration.

Centrifugation and Filtration

The sample was centrifuged at 8,000 rpm for 40 minutes at 4^oC in 500 mL centrifuge tubes. The supernatant was extracted from the centrifuge bottles and transferred into another flask. The pelleted sample was discarded. After centrifugation, gravity filtration was performed, using a filter paper gradient with pore sizes ranging from 10μm to 1μm. After the sample was filtered through the filter paper gradient, it was vacuum filtered with a pore size of 0.22 μm into a sterile bottle.

Phage Precipitation

Phage precipitation was performed with PEG and NaCl or with $Ca_3(PO_4)_2$ and Na_3PO_4. The PEG treatment was as follows. For every 200 mL of sample, 5.8 g of NaCl (Thermo Fisher Scientific, Waltham, MA) was added and 20 g of PEG-8000 (Thermo Fisher Scientific,

Waltham, MA) was added to the sterilized sample. The bottle was shaken to emulsify the mixture and was stored overnight at 4^{o}C. The next day, the sample was centrifuged at 15,000 rpm for 30 minutes using 500 mL centrifuge tubes or the smaller 100 mL centrifuge tubes, depending on the volume. The supernatant was discarded, and the pellet was resuspended with 1 mL of sterile water. If there was more than one centrifuge tube used for the same sample, then the same 1 mL of sterile water was used for all of the tubes to resuspend the pellet. Next, the resuspended pellet was pipetted into 1.5 mL microcentrifuge tubes and vortexed.

The $Ca_3(PO_4)_2$ and Na_3PO_4 treatment was as follows. The sample was treated with calcium phosphate at a concentration of 4 g/1 L, mixed for 2 hours, and settled over night. The supernatant of this mixture was then treated with 0.8 M sodium phosphate at a concentration of 36 mL/1000 mL and centrifuged at 4,000 RPM for 20 minutes. The supernatant of that mixture, which was then at a small volume, was then PEG/NaCl treated.

Dialysis

A dialysis treatment was performed. A Slide-A-Lyzer Dialysis Cassette (Thermo Fisher Scientific, Waltham, MA) and appropriately matched syringe was obtained for the 1.5 mL sample. Because the sample size was 1.5 mL, then an 18 gauge needle was required for the dialysis cassette. A 4 L beaker was filled with 3000 mL of diH_2O to create a 1:3000 ratio. An empty cassette in a buoy was placed and immersed in the diH_2O breaker to hydrate the membrane. Only 1 mL of the sample was then loaded into the cassette with air by injecting the needle in a corner port, making sure to pierce just the beginning of the membrane. After injecting the sample and air, the extra air was removed to ensure that the sample spreads across the entire membrane. The cassette was set in a buoy in the beaker, and the liquid was stirred for 2 hours. The 3000 mL was poured out and the beaker was replaced with 3000 mL of diH_2O. The cassette

was placed in this solution and dialysis was allowed to occur for 2 additional hours. The sample was removed from the cassette by injecting air double the sample volume into the membrane from a top corner. With the syringe still in the top corner and the air injected, the membrane was flipped to have the syringe corner on the bottom. The sample was removed and pipetted into a new microcentrifuge tube.

DNAse Treatment

A DNAse treatment was performed. 10X DNAse (Thermo Fisher Scientific, Waltham, MA) was used based on the volume of the sample to create a 1:1000 dilution (150 μL for every 1.5 mL). Next, proteinase K (Thermo Fisher Scientific, Waltham, MA) in a 1:200 dilution was used for a final concentration of 100 μg/mL. Then, 150 μL of DNAse and 7.5 μL of proteinase K were added to the microcentrifuge tubes. The tubes were inverted to mix and centrifuged briefly to pull down the entire sample. The samples were incubated at 37^{o}C for 30 minutes and were heat shocked at 65^{o}C for 10 minutes. The treated sample was pipetted into a 10 mL conical tube.

DNA Extraction

Before DNA extraction, a glycerol treatment was performed. The DNAse-treated sample was layered on top of 40% glycerol and placed in an ultracentrifuge for 1 hour at 35,000 rpm. The supernatant was decanted, while the pellet was re-suspended in phage buffer. DNA extraction was performed using the re-suspended pellet.

A DNA extraction was performed either using a DNA Clean-Up Resin kit or using phenol/chloroform. Two mL of pre-warmed (37^{o}C) DNA Clean-Up Resin (Promega) was added to the conical tube, and the suspension was gently pipetted up and down. 0.8 mL of water-resin-phage-DNA solution was added to two columns that were placed into catch-tubes and spun in a micro-centrifuge for 1-2 minutes. The spun-through liquid was removed, and the remaining

water-resin-phage-DNA solution was added to the top column. This was repeated until all of the water-resin-phage-DNA solution had passed through the column. Any retained impurities were washed out with 500 μL of 80% isopropanol (Thermo Fisher Scientific, Waltham, MA) in each column. The columns were spun in the micro-centrifuge for 2 minutes. The columns were then rotated 180 degrees and spun again. The column was transferred to a sterile, labelled microcentrifuge tube. A volume of 50 μL of pre-heated 80°C Tris-EDTA (TE) Buffer (Thermo Fisher Scientific, Waltham, MA) was rapidly applied to the resin column. The mixture was allowed to sit for 30-60 seconds to release the DNA. Next, the tubes were spun at 13,000 rpm at 25°C for 1 minute. The liquid in the microcentrifuge tube was kept, as this contained the eluted DNA. The DNA elution was repeated for the desired number of elutions. Nano-drop readings were obtained using the Nano-drop program. The DNA to protein ratio was also examined to determine the purity of the sample.

In addition to using resin to extract DNA, a phenol/chloroform extraction was performed to compare the efficiency. Equal amounts of phenol and chloroform were added to the re-suspended pellet isolated from the glycerol treatment. After vortexing the tubes for 30 seconds and centrifuging at maximum speed for 10 minutes, the aqueous phase was pipetted into another tube. 3M sodium acetate was added at $1/10^{th}$ of the volume of sample and vortexed. Ice-cold 100% ethanol was added at about 2-2.5 times the volume of the sample. After being stored at 20°C overnight, the sample was centrifuged at maximum speed for 5 minutes and 1 mL of room-temperature 70% ethanol was added to the sample. After another round of centrifugation, the supernatant was removed and the pellet was re-suspended.

Temperature Optimization of Polymerase Chain Reaction (PCR)

A temperature-gradient PCR was performed using the *mec* and *ccr* multiplex primers and *S. aureus* N315's DNA, ranging the annealing temperature from 50°C to 60°C. Based on that PCR experiment, an annealing temperature of 55°C was determined to be optimal temperature for both multiplex primers.

Many samples were still determined to be negative for both the *mec* and *ccr* genes, so the annealing temperature was decreased. An annealing temperature of 48°C was determined to be the most optimal temperature for both multiplex primers.

Multiplex PCR

A multiplex PCR was performed using multiplex primers for both genes. The multiplex primers were created with a detailed recipe by designating Multiplex 1 as the *mec* multiplex and Multiplex 2 as the *ccr* multiplex (Table 1) [3]. The positive *mec* control was *S. aureus* N315 (Type 2- SCC*mec*), while the negative control was *S. aureus* RN4220. The positive *ccr* control was also *S. aureus* N315 (Type 2- SCC*mec*) while the negative *ccr* control was *S. aureus* RN4220. The sample contents, for a total of a 25 μL reaction, contained 10 μL of 10x Mastermix (Qiagen TopTaq), 5 μL of DNA, 2 μL of multiplex primer, and 8 μL of sterile H_2O. The control contents, for a total of a 25 μL reaction, contained 10 μL of 10x Mastermix, 2 μL of multiplex primer, 2 μL of DNA, and 11 μL of sterile H_2O. The PCR protocol was: 92°C/2min| 92°C/2min| 48°C/1min| 72°C/2min|72°C/2min (repeat 30 cycles) 13°C/indefinitely.

Table 1. The recipe for the *mec* multiplex primers (Multiplex 1) and the *ccr* multiplex primers (Multiplex 2). Expected primer pairs and each respective PCR product size are shown. The final concentration of both Multiplex 1 and Multiplex 2 was 0.1 μM.

Multiplex 1:	Multiplex 2:
10uL- mecA1 10uL-mecA2 10uL-mecA400 fwd 10uL-mecA400 rev 10uL-mecA2_500 fwd 10uL-mecA2_500 rev 400uL- diH2O	10uL-a1 10uL-a2 10uL-a3 30uL- BC 10uL- a4.2 10uL- b4.2 10uL-yR 10uL-yF
Multiplex Products	
Multiplex 1: mecA1/mecA2- 286 bp product mecA400 fwd/rvs- 400 bp product mecA2_500 fwd/rvs- 500bp product	Multiplex 2: A1/Bc- 695 bp product A2/Bc- 937 bp product A3/Bc- 1,791 bp product A4.2/Bc- 1287 bp product Yr/Yf- 518 bp product

Inhibition PCR

An inhibition PCR test was performed to determine if any contaminants present in the sample prevented a PCR product. To perform this, 1 μL of control DNA, such as *S. aureus* N315 DNA, was added to the PCR tube, resulting in the following tube contents: 10 μL of 10x Mastermix (Qiagen TopTaq), 5 μL of sample DNA, 2 μL of multiplex primer, 1 μL of control DNA, and 7 μL of sterile H_2O.

Gel electrophoresis

The samples and controls were run on a 1% gel containing 0.5 g of agarose and 50 mL of 1X Tris-acetate-EDTA (TAE) buffer. One μL of ethidium bromide (Thermo Fisher Scientific, Waltham, MA) was added to the gel. Five μL of the 1 kb ladder was loaded into the first lane of the gel. Next, 2 μL of 8x loading dye was added to 12 μL of each PCR sample. The mixture of the 12 μL of sample and dye were then loaded into wells. The gel was run for 30 minutes at 155 V. The gel was imaged using UV light in the Bio-Rad Gel doc. If a positive was observed for a test sample, the band was gel extracted and put into a sterile microcentrifuge. The potential

positive bands for both *mec* and *ccr* for the multiplex primers were a variety of band sizes (Table 2) [3].

Sequencing

PCR positive samples were sent to Elim Biopharmaceuticals to be sequenced using universal primers (M13 forward and M13 reverse). The sequence was compared to other sequences in the BLAST database to determine the specific type of SCC*mec* element.

Table 2. The potential product sequences and sizes for the *mec* multiplex primers (Multiplex 1).

Primer Pair	Gene or region amplified	Nucleotide sequence 5'-3'	Tm	Expected product size	Description
mecA1_fwd/ mecA1_rev	*mecA*	TGAAGTAGAAATGACTGAACGTCCG/ TCTGCAGTACCGGATTTGCC	62.9	1632bp	Amplify large region of *mecA* gene
mecA2_fwd/ mecA2_rev	*mecA2*	GGAGACCAGACGTAATAGTACCTGG/ AGCATTATAGCTGGCCATCCC	66.2	1559bp	Amplify large region of *mecA2* gene
mecA400_fwd/ mecA400_rev	*mecA*	TGCTAGAGTAGCACTCGAATTAGGC/ GTTCTGCAGTACCGGATTTGCC	64.6	407bp	Amplify small region of *mecA* gene
mecA2_500_fwd/ mecA2_500_rev	*mecA2*	GCCGTGTTTATCCATTGAACGAAGC/ TGGGTTGAACCTGGTGATGTAGTG	64.6	496bp	Amplify small region of *mecA2* gene

Cloning

Any PCR and sequence positive samples were then cloned into a TOPO vector, using the TOPO TA Cloning Kit (Life Technologies). Before using the cloning kit, adenosine nucleotides were added to the PCR products. In a new PCR tube, 15 μL of PCR product, 0.5 μL of 10X Mastermix (Qiagen TopTaq), and 0.5 μL of deoxynucleotide triphosphates (dNTPs) were pipetted together. Two cycles of the same PCR protocol were performed, using an annealing temperature of 48°C. The cloning kit was then used to clone the PCR product into the TOPO vector in a 6 μL volume reaction. Four μL of PCR product, 1 μL of salt solution (1.2M NaCl, 0.06M $MgCl_2$), 0.5 μL of TOPO vector, and 0.5 μL of water were pipetted into a tube. The reaction was mixed gently, incubated for five minutes at room temperature, and placed on ice.

Transformation

Using the TOPO TA Cloning Kit (Life Technologies), NEB Turbo Competent Top10 *E.coli* cells, SOC Outgrowth Medium, pUC19 DNA, and the cloned plasmid were used to transform the plasmid into *E. coli* cells. Two tubes of 50 μL *E.coli* cells were thawed on ice. One of the tubes volume was split between two tubes for 25 μL of cells in each tube, resulting in three different tubes: one for the plasmid DNA, one for the pUC19 DNA as the positive control, and one for no DNA as the negative control. Five μL of plasmid DNA was added to the tube with 50 μL of cells, while 5 μL of pUC19 DNA was added to the tube with 25 μL of cells. All three tubes were then flicked to mix the DNA with the cells and were placed on ice for 30 minutes. The tubes were heat shocked at 42°C for 30 seconds and put back on ice for 5 minutes. 950 μL of room temperature SOC medium was pipetted into each tube, and the tubes were placed in a shaking incubator at 37°C for 60 minutes at 250 rpm. After mixing the cells thoroughly after being in the incubator, the entire mixture of each tube was spread onto warmed Xgal/Amp plates. The plates were incubated for 8-12 hours or overnight at 37°C. Colonies were observed for a white or blue color, indicating successful transformation of the plasmid or not, respectively.

III. Results

Sample Characterization

Starting in October 2012 and ending in March 2015, 65 environmental samples were collected and analyzed for *mec* and *ccr* genes (Table 3). In order to test a wide variety of samples, different types of samples were collected. Samples were categorized as water (standing water, run-off water, or marine water); animal fecal matter; soil; sewage; or agricultural food. The majority of samples that were collected were water (37%), animal fecal matter (35%), or the soil (21%). The remaining samples were sewage (5%) or agricultural food (2%). In addition to the variety of types of samples collected, the samples were mainly collected from different areas of Virginia. Many of the samples were collected in the Shenandoah Valley from local farms or large bodies of water.

Of the total 65 samples analyzed, 22% were determined to be PCR positive for either the *mec* and/or *ccr* gene. Of the 24 water samples, four samples were determined to be PCR positive for *mec* and/or *ccr* genes (Table 3). Two of the samples were PCR positive for the *mec* gene only, one of the samples was PCR positive for the *ccr* gene only, and one sample was PCR positive for both *mec* and *ccr* genes. The sample that was positive for both genes was "Newman Lake." It was predicted that this sample contained the SCC*mec* element due to the presence of both genes of interest.

Of the 23 animal fecal matter samples, five samples were determined to be PCR positive for *mec* and/or *ccr* genes (Table 3). Two of the samples were PCR positive for the *ccr* gene only and three samples was PCR positive for both *mec* and *ccr* genes, which were "C-sterile," "Goat feces," and "Compost A." It was predicted that these three samples contained the SCC*mec* element due to the presence of both genes of interest.

Of the 14 soil samples, five samples were determined to be PCR positive for *mec* and/or *ccr* genes (Table 3). Two of the samples were PCR positive for the *mec* gene only, one of the samples was PCR positive for the *ccr* gene only, and two samples was PCR positive for both *mec* and *ccr* genes, which were "Gold Compost" and "Barn." It was predicted that these two samples contained the SCC*mec* element due to the presence of both genes of interest.

Of the 4 sewage and agricultural food samples, none of the samples were determined to be PCR positive for *mec* and/or *ccr* genes (Table 3). Thus, it was predicted that none of the samples contained the SCC*mec* element due to the presence of both genes of interest.

Table 3. Sample analysis of DNA concentration, presence of the *ccr* and/or *mec* genes, and the respective PCR product size. A sample was considered positive (+) or negative (-) for a gene after confirming a PCR product after gel electrophoresis.

Sample Name[a]	**Collection Location**	**DNA Concentration (ng/uL)[b]**	***ccr* (size in bp)[c]**	***mec* (size in bp)[d]**
Newman Lake[SW]	JMU campus (Newman Lake)	4.9	+ (700)	+ (500)
Lakes-J[SW]	Virginia Beach, VA	7.3	-	-
Lake-C[SW]	Virginia Beach, VA	Unknown	+ (700)	-
Arboretum Lake[SW]	JMU campus (Arboretum)	6.9	-	-
Arboretum stream[SW]	JMU campus (Arboretum)	6.8	-	-
Indian Lakes[SW]	Virginia Beach, VA	44.7	-	-
Shenandoah Waterfall[SW]	Stanley, VA	18.4	-	-
Sample A[SW]	Harrisonburg, VA	33.1	nt	-
Sample E[SW]	Harrisonburg, VA	43.0	nt	-
Sample F[SW]	Harrisonburg, VA	48.8	nt	-
Black's Run-1[RW]	Harrisonburg, VA (downtown)	16.2	-	-
Peace Park[RW]	EMU campus	27.3	+ (700)	-
Purcell Park[RW]	Harrisonburg, VA	22.6	nt	nt
Retention pond[RW]	JMU campus	14.6	nt	nt
Waterfall[RW]	Vinton, VA	Unknown	nt	nt
Retention pond 2[RW]	JMU campus	13.2	-	-
Elkton water[RW]	Elkton, VA	Unknown	-	-

Sample Name[a]	**Collection Location**	**DNA Concentration (ng/uL)**[b]	***ccr* (size in bp)**[c]	***mec* (size in bp)**[d]
Black's Run-2[RW]	Harrisonburg, VA	5.6	-	+ (500)
Purcell Park #2[RW]	Harrisonburg, VA	39.4	-	-
Bryce Creek[RW]	Basey, VA	5.5	-	-
Stream-Dayton[RW]	Dayton, VA	15.8	-	-
Sample B[RW]	Harrisonburg, VA	42.0	nt	-
Sample C[RW]	Harrisonburg, VA	36.3	nt	-
Beta fish male[MW]	Harrisonburg, VA	Unknown	nt	nt
Goose Feces-B[AF]	JMU campus (Newman Lake)	8.7	-	-
Chicken-1[AF]	Mt. Crawford, VA	122.3	nt	nt
Chicken-2[AF]	EMU campus	21.8	nt	nt
C-sterile [AF]	Fisherville, VA	107.4	+ (600)	+ (500)
Horse-R[AF]	Harrisonburg, VA	Unknown	nt	nt
Dog-R[AF]	Harrisonburg, VA	100.2	+ (700)	-
Turkey[AF]	Unknown	Unknown	nt	nt
Goose Feces-E[AF]	Virginia Beach, VA	5.0	nt	nt
Sheep[AF]	Virginia Beach, VA	3.1	-	-
Goat feces[AF]	Elkton, VA	103.6	+ (500)	+ (400)
Compost A[AF]	Virginia	2.0	+ (500)	+ (500)
Compost B[AF]	Virginia	60.6	+ (500)	-
Horse Stall[AF]	Staunton, VA	62.9	-	-
Fecal matter A[AF]	Harrisonburg, VA	22.1	-	-
Fecal matter B[AF]	Harrisonburg, VA	12.1	-	-
Feces near Tree[AF]	Harrisonburg, VA	77.2	-	-
Dog feces[AF]	Harrisonburg, VA	34.9	-	-
Possibly feces[AF]	Harrisonburg, VA	80.4	-	-
Horse farm[AF]	King George, VA	252.0	-	-
Dry cow[AF]	Harrisonburg, VA	57.8	-	-
Wet cow[AF]	Harrisonburg, VA	12.8	-	-
Sample D[AF]	Harrisonburg, VA	60.3	nt	-
Sample G[AF]	Harrisonburg, VA	52.2	nt	-
VCU waste[SE]	Richmond, VA	10.7	-	-

Sample Name[a]	**Collection Location**	**DNA Concentration (ng/uL)**[b]	***ccr* (size in bp)**[c]	***mec* (size in bp)**[d]
Sample #2[SE]	Harrisonburg, VA	38.7	-	-
HRRSA[SE]	Harrisonburg, VA	46.8	-	-
Sand farm[SO]	Iowa	Unknown	nt	nt
Collin Schmitt[SO]	Iowa	Unknown	nt	nt
Gold Compost[SO]	Virginia	76.5	+ (600, 500)	+ (500)
Barn[SO]	Virginia	Unknown	+ (500)	+ (500)
Compost-N[SO]	Virginia	142.4	-	+ (400)
Hermit[SO]	Virginia	Unknown	-	-
Farm soil[SO]	Harrisonburg, VA (off I-81S)	17.5	nt	nt
EP home[SO]	King George, VA	5.4	+ (700)	-
Sand Farm[SO]	Harrisonburg, VA	Unknown	nt	nt
Walmart[SO]	Harrisonburg, VA	1.5	-	-
Clay[SO]	King George, VA	116.0	-	-
Dirt[SO]	King George, VA	49.0	-	-
Comp[SO]	Richmond, VA	41.1	-	-
Phage[SO]	Richmond, VA	25.6	-	+ (500)
Calf trough[F]	Mt. Crawford, VA	Unknown	-	-

[a] Different types of samples were collected, such as standing water (SW), run-off water (RW), marine water (MW), animal fecal matter (AF), sewage (SE), soil (SO), and agricultural food (F).
[b] Unknown indicated that the DNA concentration was unknown.
[c] nt designated as not tested. The 500 bp fragment amplified the *ccrC* gene. The 600 bp and 700 bp fragments amplified the *ccrA1-ccrB* genes.
[d] nt designated as not tested. The 400 bp fragment amplified the *mecA1* gene. The 500 bp fragment amplified the *mecA2* gene.

PCR results were observed after gel electrophoresis (Fig. 3). The "Phage" sample had a 500 bp band after *mec* multiplex PCR, indicating that the "Phage" sample was PCR positive for the 500 bp *mec* gene called *mecA2* (Table 3).

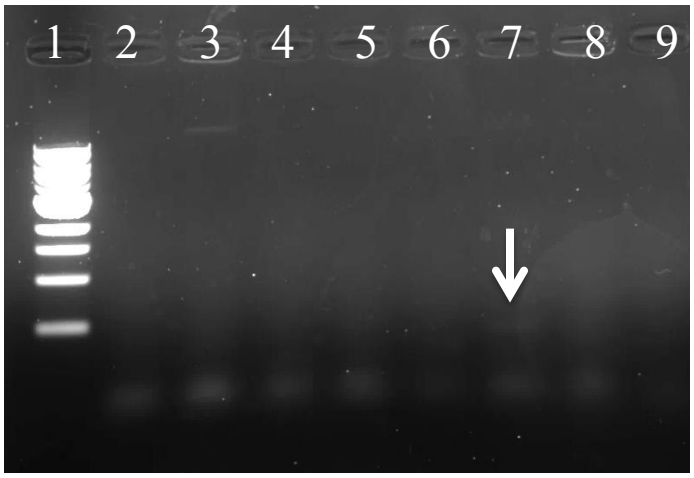

Figure 3. Gel picture of a PCR product from environmental soil sample after *mec* multiplex PCR. A PCR product was observed for the "Phage" sample (lane 7) with a 500 bp band, indicating that the "Phage" sample was positive for the 500 bp *mec* gene called *mecA2*. The gel map is as follows: 1 kb ladder (1), Clay sample (2), Dirt sample (3), Stream-Dayton sample (4), Shenandoah Waterfall sample (5), Horse farm (6), Phage (7), Comp (8), and VCU Waste (9).

The "Peace Park" sample was identified to produce a PCR product for the *ccr* gene with a 700 bp band (not shown). This positive sequence was successfully cloned and transformed into *E.coli* using a TOPO TA cloning vector. This sample was sequenced and identified to be 100% identical to *S. aureus* SCC*mec* type I.2 using NCBI Blast program.

Method Development

During the processing of samples, a large volume of them tested to be negative for both *mec* and *ccr*. Thus, other experiments were performed (termed "optimization methods"), in addition to those discussed for the "core methods," to increase the efficiency of the sample processing and ultimately result in a higher amount of PCR products produced (Table 4). A focus was placed on maximizing the amount of bacteriophage DNA present in the sample to be tested. Also, a focus was placed on maximizing potential positive samples by preventing any contamination and by determining optimal PCR protocols.

Table 4. Summary of the changes between the core methods and the optimization methods.

Step	Purpose	Rational	Core Methods	Optimization Methods
Detergent	Dissociate large molecules	Release phage from sample	Not used	Used
Centrifugation	Remove debris	Maintain phage in supernatant	8,000 rpm; 30 minutes	4,000 rpm; 20 minutes
Phage precipitation	Concentrate phage with salt treatment	Decrease total sample volume	PEG/NaCl treatment only	$Ca_3(PO_4)_2/Na_3PO_4$ → PEG/NaCl treatment
Dialysis	Remove salts from solution	Allow optimal DNAse activity	Not used	Used
DNAse treatment	Degrade bacterial DNA	Isolate only viral DNA	Used	Used
Pretreatment of Glycerol	Purify phage from the sample before extraction	Remove contaminants	Not used	Used
DNA extraction	Isolate viral DNA	Increase efficiency of extraction	DNA Clean-Up Resin extraction	Phenol/chloroform extraction
Multiplex PCR	Isolate different types of SCC*mec*	Account for the different allotypes	Used	Used

Decreased centrifugation allowed for DNA concentrations above 25 ng/μL (Table 3), compared to concentrations below 10 ng/μL with longer and faster centrifugation. The treatment of $Ca_3(PO_4)_2/Na_3PO_4$ during phage precipitation did not yield a higher concentration of viral DNA or a more pure sample (data not shown). However, this method resulted in a considerably smaller volume of sample to precipitate with PEG. This allowed for easier handling of the sample, compared to the difficulty associated with large volume samples. The pre-treatment of glycerol before DNA extraction allowed for a higher DNA concentration with less protein contamination (Table 3). The use of detergent before phage extraction, dialysis, and the phenol/chloroform extraction had no effect on the DNA concentration or efficiency of the protocol.

It was hypothesized that bacteriophage could be lost as the sample was taken through the protocol. After spiking a solution of phage buffer (Fig. 4) and spiking a sample of horse fecal matter (Fig. 5), it was determined that no step of the protocol inhibited phage or a PCR product, because the *ccrAB* gene was recovered.

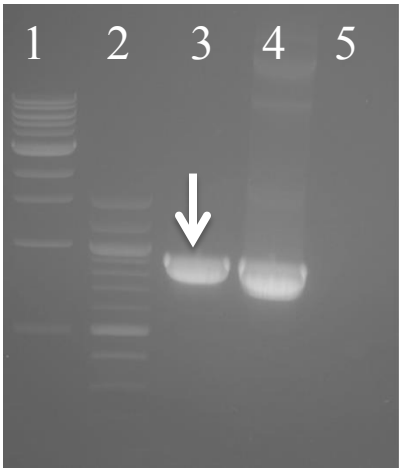

Figure 4. Gel picture of PCR control 80α pWA46 lysate after being taken through the protocol. The generation of a PCR product for the control 80α pWA46 lysate was not inhibited by the protocol. As indicated by the white arrow, there was a band observed in lane 3 at 500 bp, indicating that a PCR product was produced for the *ccr* gene. The gel map was as follows: 1 kb ladder (1), 100 bp ladder (2), spiked phage buffer + 80α pWA46 sample (3), N315 DNA-positive control for *ccr* (4), and water-negative control for *ccr* (5).

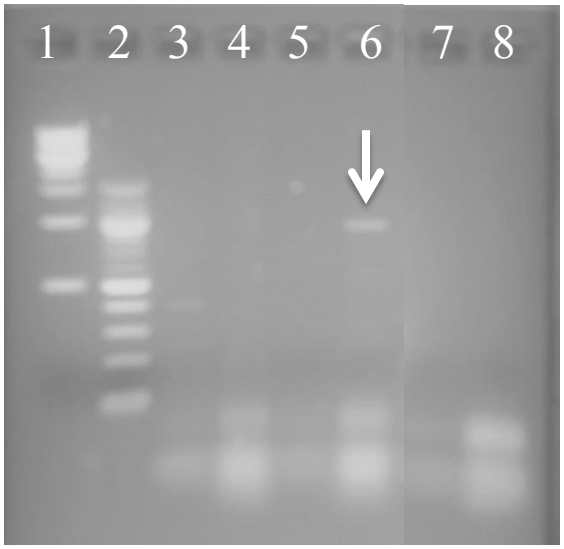

Figure 5. Gel picture of PCR of animal fecal matter sample spiked with control 80α pWA46 lysate after being taken through the protocol. The generation of a PCR product for the animal fecal matter sample spiked with 80α pWA46 lysate was not inhibited by any step in the protocol or by the environmental sample. As indicated by the white arrow, there was a band observed in lane 6 at 1 kb, indicating a PCR product produced for the *ccr* gene. The gel map was as follows: 1 kb ladder (1), 100 bp ladder (2), un-spiked horse sample *mec* (3), un-spiked horse sample *ccr* (4), spiked horse sample *mec* (5), spiked horse sample *ccr* (6), water-negative control for *mec* (7), and water-negative control for *ccr* (8).

Due to the hypothesis that there may be too little viral DNA for detection of a PCR product, 80α RN4220 pWA46 lysate was diluted by 2-fold six times. The lysate dilutions were taken through the protocol and the *ccr* gene was identified in each dilution (Fig. 6), indicating that even a low concentration or amount of DNA could be detected by PCR.

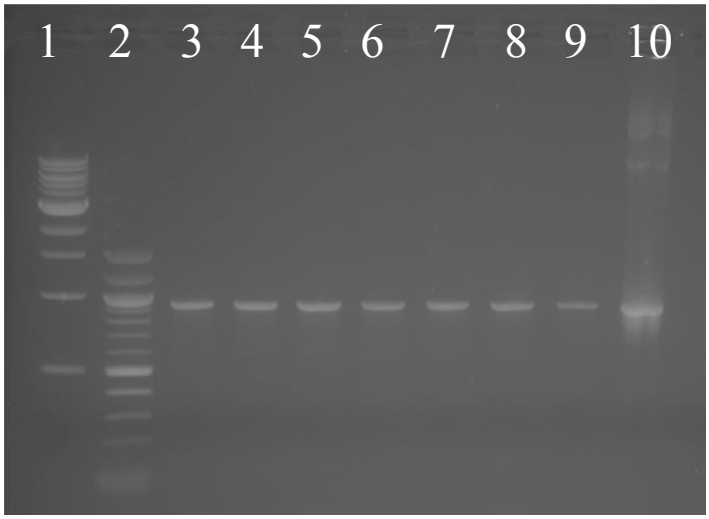

Figure 6. Gel picture of PCR of 80α RN4220 pWA46 lysate dilutions. The concentration of DNA does not affect the potential for a PCR product. The starting dilution was 108 ng/μL (lane 3) and ending dilution was 1.7 ng/μL (lane 9). Bands were observed in each dilution of the lysate at 1 kb, indicating a PCR product produced for the *ccr* gene. The gel map was as follows: 1 kb ladder (1), 100 bp ladder (2), lysate dilutions diluted by 2-fold each time (3-9), and N315-positive control for *ccr* (10).

It was determined that PCR inhibitors were present in all samples, except for one, after an inhibition PCR was performed (Fig. 7). These inhibitors were unknown and were observed to be preventing the generation of an expected PCR product from the N315 control DNA.

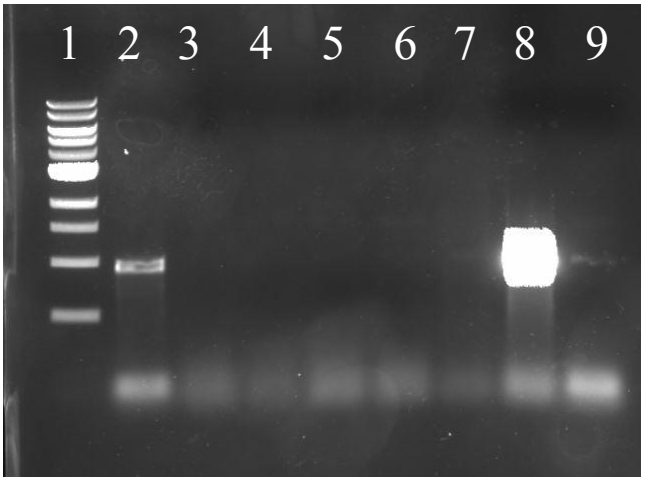

Figure 7. Gel picture of inhibition PCR of environmental samples A, B, C, E, fecal matter A, and HRRSA. There are inhibitors still present in samples that inhibit the generation of a PCR product. The gel map was as follows: 1 kb ladder (1), sample A (2), sample B (3), sample C (4), sample E (5), fecal matter A (6), HRRSA sample (7), N315-positive control (8), and water-negative control (9). This PCR and gel was performed by Colin Brooks.

IV. Discussion

As stated before, 22% of the total 65 samples analyzed were determined to be positive for either the *mec* and/or *ccr* gene by PCR. While this was a much lower gene frequency than the 100% frequency of the *mecA* gene published in the papers from Spain (7), this value still exemplified the apparent antibiotic resistance in the environment, specifically in Virginia. Not only were antibiotic resistance genes being transferred between bacteria alone in the environment (5), but it was apparent that they were also being transferred from bacteria to viruses due to the presence of these genes isolated from viral DNA.

The positive samples were isolated from water samples, animal fecal matter samples, and soil samples. However, none of these positive samples were isolated from sewage of agricultural food samples. This result indicated that this transduction was occurring in various parts of the environment and not just concentrated to only agricultural samples. A majority of the animal fecal matter was isolated from farm animals, and due to the findings of the Spanish study, it was expected to identify some samples positive for the genes of interest. Yet, soil samples collected from locations off farmland and water samples collected from rivers downstream of farms or bodies of water far from any farmland that were determined to contain the genes of interest manifested that antibiotic resistance was occurring in non-agricultural locations as well. This, again, manifested the issue of rising antibiotic resistance both in the agricultural setting and the rest of the environment.

While there were 22% of samples positive for either the *mec* or *ccr* gene, only 9% of these samples (six of the 65 total samples) were positive for both of the antibiotic resistance genes. These samples were predicted to contain the SCC*mec* element due to the low possibility that both of the *mec* and *ccr* genes would be isolated from the same sample by chance. This

result indicated that antibiotic resistance was being spread in the environment, specifically through the transfer of the SCC*mec* element. Overall, these findings indicated that methicillin resistance is increasing in the environment, which could also result in the spread of resistance to other beta-lactam antibiotics, such as oxacillin and flucloxacillin.

Method development did allow for more efficient isolation of phage and a higher amount of PCR positive samples. The use of detergent and varying salt treatments allowed for more optimal phage extraction and precipitation. Also, the 80α RN4220 control phage lysate manifested that the sample was not being inhibited by the protocol itself and that phage was not being lost during the processing. In addition, this development also manifested the reasons behind the large amount of negative samples. The manipulation of the annealing temperature from 55°C to 48°C allowed for the isolation of more PCR products. Without this finding, many environmental samples were being incorrectly identified as negative. Also, the inhibition tests manifested that unknown contaminants were preventing the generation of PCR products by an unknown mechanism. Thus, it was possible that samples that were identified to be negative could actually be identified as positive, if the contaminants were not present.

Many implications are associated with the finding of this antibiotic resistance in the environment. Specifically, people who work in the agricultural setting, whether it be on a farm or at a meat processing plant, would have to lower the daily use of the antibiotics to prevent any microorganisms from becoming resistant to them or from spreading the resistance to other bacteria. Also, if the antibiotics continued to be used, people would have to change the antibiotic type and dosage due to the antibiotic resistance that would result. If those that work in agriculture still desire to use antibiotics, they would have to use antibiotics other than those in the class of beta-lactam antibiotics due to the apparent methicillin resistance found in this study.

There were limitations to this study that could have affected the findings. First, only five different samples types were used. It was possible the antibiotic resistance genes of interest were found at a higher frequency in samples other than those that were tested, such as meat from animals on a farm or soil from the bottom of rivers downstream of farms. Second, this study's sample collection was very limited to Virginia. It was possible that collecting samples from a larger geographic area would have yielded a higher frequency of PCR products. Third, in terms of analysis, many of the samples had a DNA concentration less than 100 ng/μL. While concentration was determined to not be an issue for detection of the genes *mec* or *ccr* (Fig. 4), the low concentration presented difficulty when sequencing the PCR products. Sequencing typically required a large volume of highly concentrated DNA, so many samples could not be sequenced to determine the specific type of *mec* or *ccr* gene isolated.

Future work would be to collect environmental samples from outside the state of Virginia to determine if the geographic location of sample collection will affect the findings. In addition, further analysis into the inhibition tests is needed to determine the specific contaminant that is preventing a PCR product. Also, further analysis of the "Peace Park" sequence is needed to better understand the SCC*mec* element and how it is transduced from *S. aureus* bacteria to *Staphylococcus* bacteriophages.

Despite the challenges and limitations to this study, the findings manifested the presence of methicillin resistance in environmental samples from different areas of Virginia. Also, it was evident that this resistance is being transferred by transduction due to the isolation of both *mec* and *ccr* genes from viral DNA. Those that work or perform research in the agricultural field need to be aware of these important scientific findings and understand their implications on the agricultural industry and the environment as a whole.

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