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Direct capture of plasmids conferring resistance to beta-lactam antibiotics from agriculturally-impacted stream sediment

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Direct Capture of Plasmids Conferring Resistance to Beta-Lactam Antibiotics from Agriculturally-Impacted Stream Sediment

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Abstract

Streams harbor large numbers of native and introduced bacteria, which can be both recipients and donors of antibiotic resistance genes on mobile elements. Transmissible, plasmid- borne resistance to expanded-spectrum cephalosporins and fluoroquinolones is of increasing concern in clinical settings, but is rare in natural environments, such as streams and soils. Using a method developed in our laboratory, a rifampicin-resistant strain of *Escherichia coli* was used to capture, without culturing, two plasmids allowing growth on trypticase soy agar supplemented with the beta lactam antibiotics ampicillin and ceftazadime. Plasmids were captured directly from stream sediment taken from Muddy Creek, a stream heavily impacted by agricultural runoff. Transconjugants were also tested – using a modified "Stokes" disk diffusion method – for resistance to tetracycline, gentamycin, ciprofloxacin and ceftriaxone. One transconjugant, originally isolated from a rifampicin/ceftazidime plate, was resistant to ciprofloxacin, ampicillin, and tetracycline, in addition to ceftazadime. A second transconjugant, originally isolated from a rifampicin/ampicillin plate, was also resistant to ciprofloxacin and tetracycline, in addition to ampicillin. To confirm that the transconjugants were indeed the rifampicin-resistant capture strain, they were fingerprinted using PCR amplification of BOX repetitive sequences. The presence of plasmids was confirmed via a plasmid preparation method specifically developed in our lab for large plasmids; the plasmids appear to be in the 50-100 kb range. The recovery of transmissible plasmids encoding resistance to late-generation, human therapeutic antibiotics, such as ceftazadime and ciprofloxacin, from streams is surprising since such antibiotics are not approved for use in agriculture. It also suggests the presence of a reservoir – of unknown size and composition – of transmissible antibiotic resistance genes in stream sediment bacterial communities impacted by agricultural runoff.

Introduction

Bacteria with antibiotic resistance potential have been in existence for at least 30,000 years, according to researchers who have extracted samples from Yukon permafrost (Wright *et.al*, 2011), and probably much much longer. Antibiotics are known today as chemical substances that kill or interfere with the growth and development of bacteria. They have been widely used over the past 70 years. In fact, antimicrobial agents have been reducing the number of deaths and occurrence of infectious diseases since the 1940s (CDC, 2010).

If prescribed correctly, antibiotics are very beneficial as they help the immune system to fight off harmful bacterial strains. However, antibiotics have frequently been overprescribed and incorrectly used. This has led to a rapid increase in the prevalence of drug-resistant microorganisms both in clinical settings and in the environment. The continuous increase of antibiotic-resistant bacteria has become a major public health threat as some bacterial strains have become multi-resistant and thus untreatable (Andersson, 2003).

Antibiotic resistance is spread primarily as a result of horizontal gene transfer between bacteria. There are three different ways bacteria may acquire resistance. Many bacterial species inherit resistance genes "vertically" from their predecessors. Other times, bacteria become resistant through random genetic mutations that spontaneously create a new resistance trait and/or strengthen an already existing one. Most frequently, resistance occurs as a result of the transfer of genetic resistance information between the same or different species. This happens through elaborate mechanisms via a variety of gene transfer systems, including but not limited to bacterial conjugative plasmid systems, bacteriophages, integrons and transposons (Bennett, 2008). Horizontal gene transfer may occur by three different processes: transduction, transformation or conjugation. Transduction is a process where bacterial genes are transferred

from one cell to another by means of viral vectors (Griffiths, 2000). Transformation occurs when the cell takes up free DNA from the environment. The process is dependent on specific genes located in the host cell that direct the uptake and the integration of the acquired foreign DNA (Lorenz, 1994). The ability of a cell to take up free DNA is known as "competency". Competency can be present all the time, at certain times in the life cycle, or promoted by the environment, depending on the species of bacteria (Davison, 1999). It can be induced under laboratory conditions or it can occur naturally under specific environmental conditions.

Conjugation is perhaps the most prevalent process of the three methods of horizontal gene transfer (Top *et al.*, 1994). It requires physical contact between the bacteria engaged in the process. Both the "donor" and the "recipient" bacteria may contain one or more conjugative plasmids. Conjugative plasmids may be self-transmissible – possessing all the necessary genes to transmit themselves to another bacterium – or mobilizable, in which a self-transmissible plasmid must be present in the same cell to facilitate transfer. *Tra* and *oriT* genes are both found on self-transmissible plasmids. The *tra* gene enables the bacterium to form a mating pair with another bacterium, whereas the *oriT* gene is responsible for determining where plasmid transfer is initiated. Mobilizable plasmids differ from self-transmissible plasmids in that they lack the *tra* genes for self-transmissibility. However, mobilizable plasmids do have the ability to undergo conjugation as long as the host cell also contains a transmissible plasmid, which provides *in trans* the necessary transfer functions for both the transmissible and the mobilizable plasmids (Griffiths, 2000).

Plasmids are normally studied by first isolating plasmid-containing bacteria from a given environment; plasmids can then be studied within the host cell or isolated and transferred to other host cells. This approach, sometimes called "endogenous" plasmid isolation, only allows

for the study of plasmids from *cultured* cells. However, since the vast majority of microorganisms in their natural habitats (particularly environmental habitats such as soils and waters) are not yet culturable, an endogenous approach of necessity excludes most plasmids from study. So-called "exogenous" plasmid isolation, on the other hand, allows for the isolation of plasmids from both "culturable" and "not yet culturable" cells (Smalla *et al.* 2000).

The exogenous plasmid isolation method was developed by Fry and Day in 1990. It samples the plasmid population by allowing bacteria to transfer plasmids to a known recipient via conjugation (Fry & Day, 1990). This technique is very efficient because it allows for sampling from uncultured bacterial populations in stream, soil and sediment environments. Theoretically, sampling uncultured bacterial populations allows for the capture and isolation of a greater, more diverse population of plasmids. The only disadvantage of this method is that not all plasmids are able to conjugate with the recipient and not all genes on the plasmids may be expressed in the recipient host cell.

One major factor, which makes bacteria resistant to beta-lactam antibiotics such as penicillins and caphalosporins, are bacterial beta-lactam enzymes. Extended-spectrum beta-lactamase (ESBL) enzymes are a group of enzymes that can hydrolyze extended-spectrum cephalosporins, making these enzymes a primary resistance concern (Philippon *et al.*, 1989). Extended-spectrum beta-lactamases, produced by primarily be members of the Enterobacteriaceae, have become increasingly prominent and relevant since the late 1990s (Ewers et. al, 2012). While ESBL bacterial strains were initially observed only in humans, it has been shown that ESBL-producing strains of *E. coli* are becoming more prevalent in food-producing animals (Ewers *et al.*, 2012). Despite certain regulations put in place to decrease the amount of multi-resistant bacteria, such as the EU-wide ban of growth promoters, there is still a

lack of understanding about the course of transmission for the ESBL-producing bacteria between animals and humans (Ewers *et al.*, 2012). In order to further investigate the mode of transfer of resistance to beta-lactam antibiotics and cephalosporins, it is beneficial to explore the transfer of resistance plasmids derived from a natural, environmental setting, but within the boundaries of the laboratory.

As antibiotic resistance spreads rapidly in the environment and in clinical settings, it is important to be able to trace and follow the transmissibility among bacteria. One of the leading hypotheses in our laboratory is that native and introduced bacterial populations in stream sediments harbor an ever-expanding reservoir of resistance genes, which have the ability to recombine and subsequently transfer from native to transient bacterial populations. It is believed that both native and introduced bacteria can exchange genetic information that can lead to increased levels of antibiotic resistance. What is more, resistance genes may be carried on genomic islands where they can recombine and even produce a multiresistance phenotype that might then be transferred to non-native bacteria, including animal and potential human pathogens.

As the use of antimicrobials in agriculture is increasing, antibiotic resistance spread in natural environments is increasing as well. This trend poses a threat to the way bacterial infections are treated in clinical settings because some bacterial species have acquired resistance to multiple drugs, which makes them hard or impossible to treat (Shea *et.al*, 2003). This study investigated and characterized the presence of beta-lactam resistant plasmids in agriculturally-impacted stream sediment. By studying the transmissibility of antibiotic resistance in bacteria indigenous to freshwater ecosystems, we were able to gain a better idea of the exchange and spread of genes in stream environments. In addition, in a given water ecosystem, changes related

to increased antibiotic resistance spread caused by different factors such as, but not limited to, increased livestock antibiotic use, have shown to be often influenced by human action (Baquero *et.al,* 2008). Therefore, this study has implications in both clinical and ecological fields, and sheds light on the potential transfer of resistance genes that originated in a native stream setting.

In order to select resistance plasmids acquired from stream sediment, three antibiotics were used – ampicillin, cefotaxime, and ceftazidime. The World Health Organization classifies these as critically important antibiotics used in human medicine (Collignon *et.al,* 2009). For instance, ampicillin is a beta-lactam antibiotic from the aminopenicillin family, used for treating a wide range of bacterial infections, such as bronchitis, pneumonia, ear, lung and skin infections. Cefotaxime and ceftazidime are third generation cephalosporins, often intravenously administered in patients to eliminate bacteria that cause bone, joint, stomach, blood, lung, skin and urinary tract infections. (USDHHS, 2013). Isolating plasmids encoding resistance to any of these antibiotics from stream-obtained bacterial populations would raise the problematic question of resistance determinants mobilizing within stream sediment.

Materials and Methods

Field Sites and Sample Collection

The study used samples collected from Muddy Creek, a shallow stream that originates in the Allegheny Mountains of Virginia and flows east into the Shenandoah Valley. The stream flows through heavily-utilized cattle pastures, farms, and near a large poultry processing plant before emptying into the Dry River and North River, which are tributaries of the Shenandoah River. The average creek depth and width fall between 0.1 to 0.25 m and 2.5 to 5 m, respectively. The lower regions of the creek contain extremely high levels of fecal runoff as well as runoff from the nearby poultry plant. The concentration of fecal coliform counts often exceeds 16,000/100 ml, which classifies the creek as one of Virginia's most impaired water bodies, according to the Virginia Department of Environmental Quality (Workgroup, 1999).

Samples were collected from the lower region of the creek where there is an influx pipe from a nearby poultry plant. Sterile 50 mL Falcon® tubes were used for sampling of both water and sediment, at three different locations in the stream: 30 feet upstream of the influx pipe, at the influx pipe, and 30 feet downstream of the influx pipe. Sediment was collected in order to capture plasmids from bacterial populations that are in higher abundance and may have been residing longer in the stream compared to those in the water column. For the sediment sampling, areas of fine sediment were used. The top layer of 2-5 mm of sediment was removed by using a flamed trowel. Falcon tubes were then inserted with gloved hands top down in order to avoid picking up excess water. Next, sediment was scooped and any excess water in the tube was poured out. For the water sampling procedure, an area of flowing water with few visible floating particles was used. The conical tube lids were left on until the tube was halfway into the water in order to avoid collecting surface water. The tube was tilted to collect the moving water, and re-

capped while in the water, again to avoid surface water collection. Once collected, the samples were transported to the lab on ice and refrigerated until time of use. Samples were utilized within 24 hours of collection.

Capture Strain

An *Escherichia coli* strain, labeled LA61, was used to capture the plasmids for the first part of this project. The LA61 strain was originally isolated by Dr. Elizabeth Alm of Central Michigan University from a beach in Michigan, and later on developed in the Herrick lab to be plasmid-free and rifampicin resistant. The absence of plasmid in the strain allowed for the selection of putative transconjugants once the mating occurred. Trypticase Soy Agar (TSA) was used as a basic nutrient agar medium for growing, isolating, and maintaining bacteria. In addition, growth and lactose fermentation on MacConkey agar was used to help differentiate LA61.

Determination of Antibiotic Concentrations Used for Exogenous Isolation

Initially, the minimum inhibitory concentrations (MICs) on trypticase soy agar (TSA) and in trypticase soy broth (TSB) was determined for the antibiotic cefotaxime (cef) in order to determine the optimum concentration of antibiotic to be used for exogenous capture. Increasing concentrations of the antibiotic ((1ug/ml, 2ug/ml, 4ug/ml, 8ug/ml, 16ug/ml, 32ug/ml and 64ug/ml) were spread on separate tryptic soy agar (TSA) plates and inoculated with the recipient strain *E.coli LA61* for 24 hours at 37°C. The plate with the lowest concentration, which demonstrated growth, was the one that determined the MIC of strain LA61 for cef. The same procedure was performed with TSB tubes.

For the ceftazidime (caz) and ampicillin (amp) concentrations, MICs were determined from previous studies: the MIC of cef was 2 ug/ml and of amp was 50 ug/ml (Salmon, 2000).

Exogenous Plasmid Isolation

In order to release cells from the sediment, 10 g of wet weight sediment were mixed with 90 mL of 0.1% sodium pyrophosphate (NaPyro, pH 7.4). After allowing the sample to settle for 5 minutes at room temperature, 1 mL of the supernatant was pipetted into a 1.5 mL Eppendorf tube, centrifuged at 5,800x g for 8 minutes and washed in phosphate saline buffer (PSB, 80mM Na_2PO_4 (dibasic), 70mM KH_2PO_4 (monobasic), 145 mM NaCl and adjusted to pH7) two consecutive times. The cells were then resuspended in 0.5 mL of PSB and mixed with 0.5 mL of recipient strain *E.coli* LA61. *E. coli* LA61 was cultured overnight in TSB at 37°C at 180 RPM. Once mixed together, the mixture of cells and recipient *E .coli* were vortexed, and then 200 µL were pipetted onto a 0.45 µm 47 mm round filter on a TSA plate and incubated at 37°C for 24 hrs. The filter was then carefully transferred to a 50 mL conical tube, containing 10 mL PSB, and the tube vortexed to release the cells from the filter. Potential transconjugants were then plated onto TSA containing rifampicin (rif) and either cefotaxime (cef), ceftazidime (cfx), or ampicillin (amp). As negative controls, cells released from the sediment were plated on a TSA + cef or cfx or amp plate, and on TSA + rifampicin. The recipient, LA61, was plated on TSA + cef or cfx or amp as a negative control and on TSA + rif as a positive control.

Plasmid purification and visualization

In order to isolate and visualize plasmid DNA, a large plasmid isolation procedure developed in our laboratory was performed on transconjugants. Resistant cells were cultured overnight in sterile TSB tubes at 37°C. One and a half milliliters of the turbid broth were centrifuged at 10,000x g for 5 min and the supernatant removed. The cell pellet was resuspended in 100 ul of alkaline resuspension buffer (10 mM EDTA, 50 mM dextrose, 10 mM Tris-Cl, pH 8.0). Two hundred microliters of 0.2 M NaOH/1% SDS were added to lyse cells. The process

was continued by inverting the tube and letting it sit at room temperature for approximately 5 minutes. One hundred and fifty micro liters of ammonium acetate were added to denature the DNA and reduce the pH, and 150 ul of chloroform were added to denature proteins. This mixture was then centrifuged at 16,000x g for 10 min and the supernatant removed and added to 30% polyethylene glycol 8000/1.5M NaCl (PEG) solution in order to separate the plasmid DNA from the chromosomal DNA matrix. The PEG and DNA were put on ice for 15 min and then centrifuged at 16,000x g for 10 min. The supernatant containing the chromosomal DNA was removed and the plasmid DNA pellet was resuspended in 100 ul sterile ddH_2O and stored at -20°C .

The plasmid DNA was visualized using agarose gel electrophoresis by adding 10 ul of plasmid DNA with 2 ul of 6X loading dye (Promega, Madison, WI) on a 0.7% agarose gel and running at 90V for 2 hours. Once the run was complete, the gel was stained with ethidium bromide for about thirty minutes immediately followed by destaining in deionized water for ten minutes. A photograph of the gel was taken electronically using a BioRad ChemiDoc gel documentation system.

Rep-PCR Fingerprinting Using BOX Primers

Repetitive sequence DNA fingerprinting using BOX primers ("BOX-PCR"; Rademaker *et. al.,* 1998) was used to verify that transconjugants were indeed strain LA61. Strain LA61 was used as a positive control. An inoculating needle was used to pick up a small number of cells from each sample grown overnight at 37°C on a TSA + antibiotic plate. Cells were added to 10 µL of diH_2O in a sterile 0.2 ml PCR Eppendorf tube and lysed by denaturation in a thermocycler at 95°C for 5 mins. Three microliters of lysed cells were then added to a separate 0.2 ml PCR Eppendorf tube, containing 5 µL of Premix G (Epicentre Technologies, Madison, WI) and 2 µL

of PCR master mix. The master mix contained 1.3 µL of BOX primer, with sequence 5'-CTACGGCAAGGCGACGCTGACG-3' (Sigma Genosys, Woodsland, TX), 0.1 µl Failsafe enzyme mix (Epicentre Technologies, Madison, WI) and 0.6 µl of sterile diH_20. The PCR reaction was then incubated at 95°C for 2 mins. followed by 30 cycles of 94°C for 3 s, 92°C for 30 s, 50°C for 60 s, and 65°C for 8 mins, followed by one cycle of 65°C for 9 mins. PCR products were refrigerated at -20°C until visualized.

Samples were visualized using agarose gel electrophoresis. Ten microliters of the amplified DNA were loaded into a 1% agarose gel with 2 µL of 6x loading dye. The gel was then run at 60 V for 2.5 hours. 0.5% ethidium bromide was used to stain the gel for approximately 15-20 mins and diH_2O were used to destain for about 15 minutes. Band patterns were used to verify that the transconjugants were the LA61 *E. coli* recipient strain, as shown in the results section.

Modified Stokes Method – Antibiotic Susceptibility Test

Mueller-Hinton plates were divided into three sections, with LA61 plated on the outsides of the plate and the possible transconjugant plated in the middle (see Figure 1). Antibiotic disks were placed on both marker lines spaced out equally and in a way allowing sufficient space for measuring zones of growth inhibition. This method, a modification of the Stokes method for assessing antibiotic susceptibility used in the United Kingdom (Stokes, 1972), allows for direct comparison of antibiotic zones of inhibitions of both the recipient strain and the transcojugant. A decrease in zone inhibition size for an antibiotic relative to that for the recipient strain alone is indicative of a possible decrease in susceptibility to the antibiotic. The zone of inhibition was measured by the use of calipers from the edge of the disk to the edge of the bacterial growth. A difference of $\geq$3mm was used as a conservative cutoff indicating resistance (Collins, 1989).

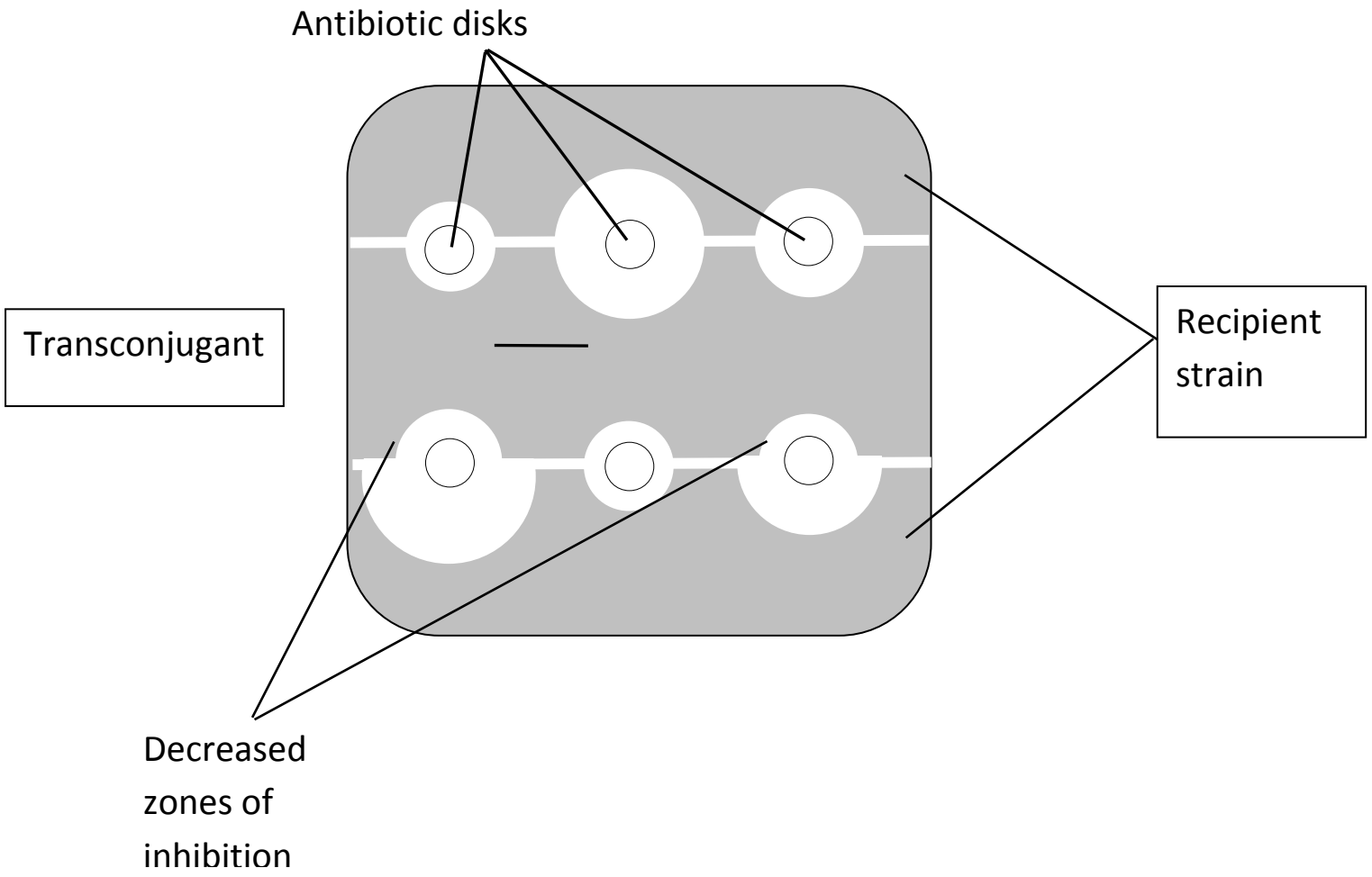

Figure 1. Stokes method for the determination of decrease in antibiotic susceptibility, adapted for comparing transconjugant to recipient strains. Two parallel lines were drawn on a plate containing Mueller-Hinton agar and the recipient strain swabbed along the outsides of the plate and the transconjugant to be tested along the center of the plate. Antibiotic-containing disks were placed along the lines and, after incubation, the radius of total clearing measured with an electronic caliper. For this study, a difference $\geq$ 3 mm in the radius of clearing between the recipient and transconjugant was defined as representing the breakpoint for resistance {Collins, 1989).

Results

Sediment samples were obtained from Muddy Creek in October 2012 in a location downstream of an efflux pipe that is coming from a nearby poultry plant. The cells were separated from the mud and released in sodium pyrophosphate. Next, they were mixed with a rifampicin resistant strain of *E.coli* – LA61, which was used as the recipient organism. The resultant mixture of cells and LA61 was then grown overnight on trypticase soy agar (TSA) plates previously prepared in the lab. Following a 24-hours incubation period, the cells were plated on modified TSA plates. Three types of modified TSA plates were created – one amended with rifampicin and ampicillin, a second type with rifampicin and ceftazidime, and a third one with rifampicin and cefotaxime. Cells capable of growing on these TSA plates were picked as putative transconjugants and processed for further verification. There were multiple colonies on all three kinds of amended TSA plates. These putative transconjugants were streaked on TSA plates before they were verified for plasmid capture into the LA61 recipient.

MacConkey agar and Eosin Methylene Blue agar were the selective media used to initially verify that the transconjugants were the recipient *E.coli* LA61 strain. MacConkey agar is a selective medium which inhibits the growth of Gram-positive organisms because it contains bile salts, peptone, lactose, neutral red and crystal violet. Organisms that are Gram-negative lactose fermenters produce acid products, which lower the pH of the media below 6.8. In addition, the nearby bile salts precipitate. The resultant colonies appear pink with pink halos (Leboffe, 2010). When the putative transconjugants were plated on MacConkey agar, all nine colonies on the plate appeared to have pink color and halos. Eosin methylene blue agar was the other selective medium used for verification. It contains eosin and methylene blue dyes that inhibit the growth of Gram-positive organisms. In addition, the medium differentiates lactose

fermenters that produce acid products by changing its color. Generally, *E.coli* has a strong acid production, which turns in as a result a metallic green sheen growth (Leboffe, 2010). From the plated organisms, ten colonies appeared to have metallic green growth. After confirming that the results of the two selective media verification tests were positive, nine out of the ten transconjugants were chosen as possible transconjugants for further verification and processing.

BOX rep-PCR was used as another verification method since the selective media could not identify putative transconjugants as specifically strain LA61. The BOX rep-PCR amplifies repetitive intergenic sequences of DNA, which helped in comparing repeating sequences between the transconjugants and the LA61 strain. The method was utilized on seven transconjugants in order to determine which of them had similar banding patterns to that of the original LA61 strain. Four putative transconjugants were selected due to the similarity of bands to LA61. These four transconjugants were located in lanes 5, 8, 10, and 11 on the gel, and consequently were named BL1, BL2, BL3, and BL4, respectively.

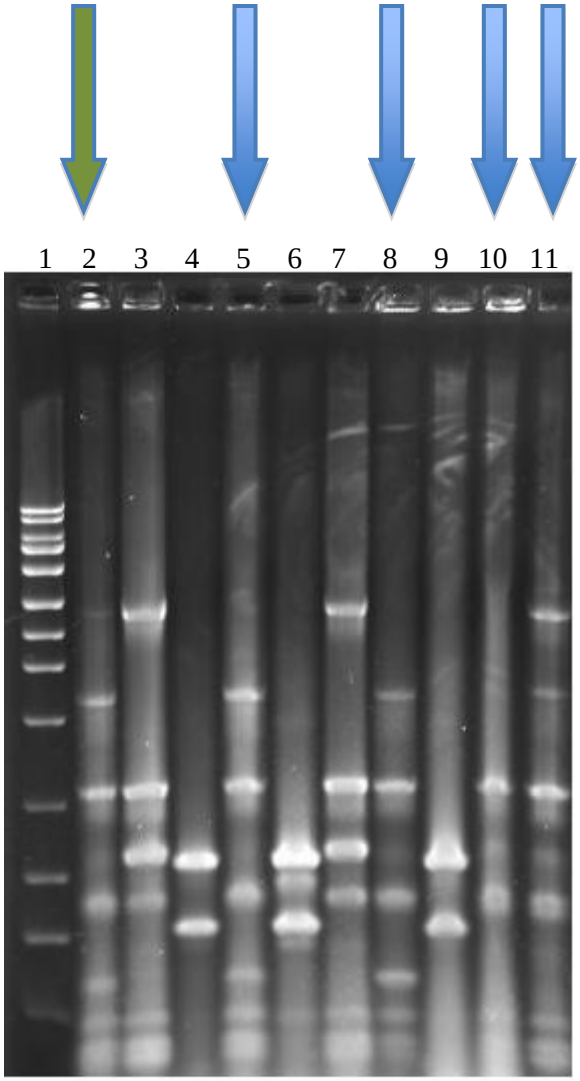

Figure 2. Gel results from BOX rep-PCR. Lane 1 - 1kb ladder, Lane 2 - LA61 recipient, Lanes 3-11 - putative transconjugants isolated from combination antibiotic TSA plates. The green arrow points to the LA61 recipient to which the samples were compared to. The four blue arrows point to the samples that showed similarities to the LA61.

In the next step of the project, the presence of plasmids in the transconjugants was investigated. Plasmids in transconjugants BL1, BL2, BL3, and BL4 were confirmed by performing a plasmid preparation. The first plasmid preparation gel that was run on all four isolates indicated no plasmid presence in BL1 and BL2. As a result, these two isolates were excluded from the study. Another 0.7% agarose gel was run on BL3 and BL4 to demonstrate the presence of plasmid bands (see Figure 3). On the same gel both negative and positive controls were included. The negative control (recipient strain LA61) contained no plasmid therefore, when visualized on the gel, it contained only one distinct band, whereas the positive control (a *Pseudomonas aeruginosa* strain from our laboratory) showed at least three distinct bands indicating the presence of multiple plasmids. Transconjugants BL3 and BL4 each had one band of plasmid DNA, which is seen above the chromosomal DNA (indicated by blue arrows).

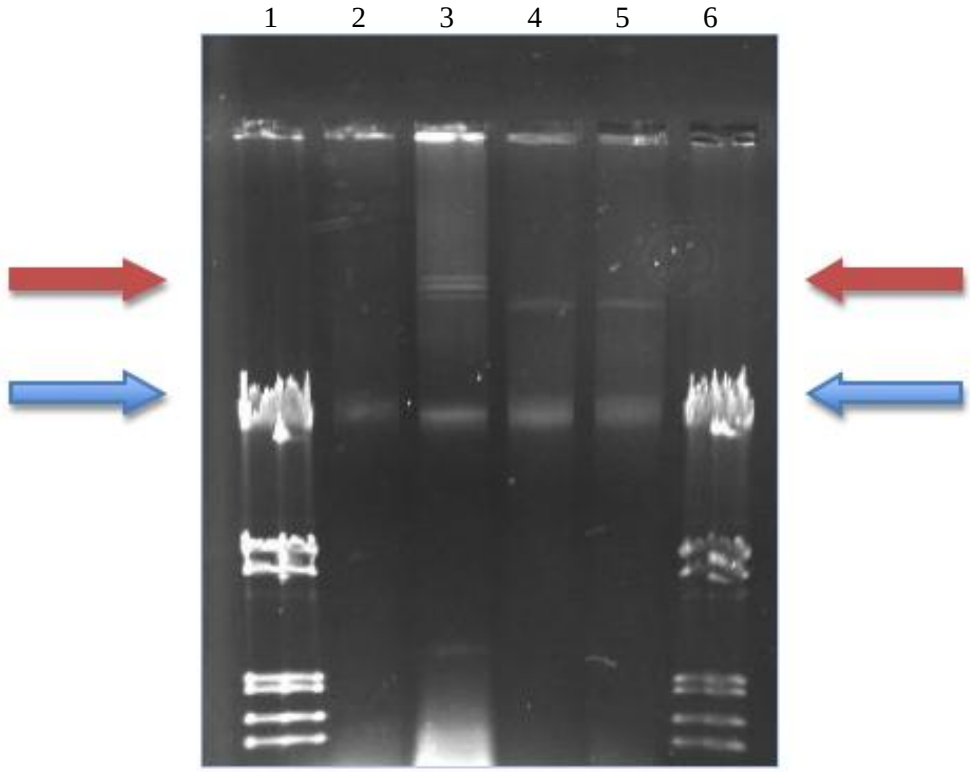

Figure 3. Gel visualizing the results from the plasmid preparation. Lanes 1 and 6 – 100 kb ladder, lane 2 – negative control - LA61 strain, lane 3 – positive control – *Pseudomonas aureginosa,* lane 4 – BL3 transconjugant, lane 5 – BL4 transconjugant. Red arrows indicate plasmid DNA. Blue arrows indicate chromosomal DNA.

Once confirmed through plasmid preparation and gel visualization, the transconjugated plasmid sample isolates pBL3 and pBL4 were screened for resistance to multiple antibiotics by using the modified Stokes method (refer to Methods and Materials section). While similar to Kirby-Bauer, the modified Stokes method has one major advantage – it allows for simultaneous comparison of antibiotic resistance profiles of the recipient LA61 strain and the transconjugants BL3 and BL4. The antibiotics tested were ampicillin, cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, gentamicin, imipenem, kanamycin, piperacillin, streptomycin, tetracycline, tobramycin, and piperacillin/tazobactam. Zones of inhibition for the transconjugants and the

original recipient were measured from the edge of the disk to the first visible growth. The difference was calculated by subtracting the BL3 or BL4 zone from the LA61 zone. A difference of $\geq$ 2.5mm was considered a measurable decrease in antibiotic susceptibility whereas a difference of $\geq$ 3mm was considered resistant (Collins *et.al*, 1989). As shown in Table 1, the negative numbers represent values indicating that the zone of inhibition of the recipient LA61 strain is smaller than the transconjugant zone of inhibition.

Plasmid pBL3 was found to be resistant to ampicillin, ceftazidime, piperacillin, and tetracycline. Considering that plasmid pBL3 was first isolated from a rifampicin/ceftazidime plate, the presence of ceftazidime resistance is supported. In addition, plasmid BL3 exhibited decreased susceptibility to two clinically-significant antibiotics – the broad spectrum ciprofloxacin and streptomycin. Plasmid pBL4, originally isolated from a rifampicin/ampicillin plate, exhibited resistance to ampicillin, piperacillin/tazobactam, tetracycline, and tobramycin.

Table 1. Differences in zone of inhibition measurements between two transconjugants isolated on a spectra of beta-lactam antibiotics and the recipient, *E. coli* LA61. The modified Stokes Method was used (see Materials and Methods section).
S = Susceptible, R = Resistant, DS = Decreased Susceptibility

Antibiotic Disk	BL3[1] (mm)	BL4[1] (mm)
Ampicillin	3.5 (R)	3.0 (R)
Cefotaxime	0.25 (S)	2.0 (S)
Ceftazidime	3.0 (R)	0.5 (S)
Ceftriaxone	-0.5 (S)	0.25 (S)
Ciprofloxacin	2.7 (DS)	2.0 (S)
Gentamycin	0.5 (S)	-0.25 (S)
Imipenem	-4.0 (S)	-2.0 (S)
Kanamycin	0 (S)	0 (S)
Piperacillin	3.0 (R)	2.5 (DS)
Piperacillin/Tazobactam	0 (S)	3.5 (R)
Streptomycin	2.5 (DS)	2.0 (S)
Tetracycline	4.5 (R)	4.0 (R)
Tobramycin	2.0 (S)	3.25 (R)

[1] represents the mean of two measurements.

Discussion

In this study, exogenous plasmid capture method was used to isolate transmissible beta-lactam resistance plasmids from bacteria living in fresh water sediment. The spectrum of resistance of these plasmids was wider than expected. Besides resistance to common agricultural antibiotics, resistance to clinically-significant antibiotics was found as well.

Generally, antibiotics are used in agriculture for two main purposes – to treat and prevent diseases and to promote faster growth in livestock used in farms for the production of dairy and meat products. It has been suggested that approximately 40% of all antibiotics are used exclusively in agriculture (Hostetler, 2000) although other estimates are much higher (Florini *et.al*, 2005). This use may lead to an increased presence of resistance genes in rivers and streams near production plants and agricultural grazing lands. This may result in reservoirs of resistance, particularly in bacterial populations inhabiting sediments, which can potentially be transferred via horizontal gene transfer to native and introduced water organisms (Baquero *et.al*, 2008). The resistance profiles of the two plasmids found in the given study indicate a disturbing trend where transmissible plasmids encode resistance not only to agricultural antibiotics, but also to late-generation, clinically used beta-lactam antibiotics, such as piperacillin and third generation cephalosporins. This suggests that plasmids encoding resistance to human clinical antibiotics may be common in the microflora of streams and creeks impacted by agricultural activity. In addition, such resistant plasmids raise questions generally about the use of antibiotics in agriculture, which requires further, more in-depth investigation.

In the given study, samples were collected from Muddy Creek sediment, close to Harrisonburg, VA. The creek is known to be affected by cattle farms and a poultry processing

plant located right by the creek. The sediment samples were used as a donor for exogenous plasmid capture into a lab-developed rifampicin-resistant *E.coli* recipient – LA61. Transconjugants, marked as BL3 and BL4, were isolated on ceftazidime/rifampicin and ampicillin/rifampicin selective media, respectively. They were confirmed to be conjugated *E.coli* LA61 recipients by standard media and via BOX rep-PCR, as shown in Figure 2. Both transconjugants were found to contain large (over 50 kB) plasmids (Figure 3), and their spectrum of antibiotic resistance was established through the modified Stokes method (refer to Materials and Methods). The plasmid isolated from the rifampicin/ceftazidime plate, pBL3, was found to be resistant to ampicillin, ceftazidime, piperacillin and tetracycline. In addition, pBL3 had a decreased susceptibility to ciprofloxacin and streptomycin (see Table 1). The plasmid isolated from the rifampicin/ampicillin plate, pBL4, demonstrated resistance to ampicillin, tetracycline, tobramycin, and piperacillin/tazobactam. It also exhibited decreased susceptibility to piperacillin, just as pBL3 (see Table 1).

The results from this study demonstrated that plasmid pBL3 and pBL4 exhibit resistance or decreased susceptibility to multiple clinically-significant antibiotics, which are used in preventing and treating infections in humans. Third-generation broad-spectrum cephalosporins are antimicrobial agents widely used in a variety of clinical cases. Cefotaxime is primarily used in infections caused by Gram-positive bacteria (Cunha, 1995). On the other hand, ceftazidime and cefoperazone (not investigated in this study) are some of the only third-generation antimicrobials capable of providing antipseudomonal protection (Cunha, 1995). In addition, ceftazidime is used in treating *Salmonella*-caused meningitis (Collignon *et.al,* 2009). Ceftazidime resistance genes are reportedly transmitted through *E.coli* and *Salmonella* organisms in a trend from non-human to human sources (Collignon, 2009). Third and fourth generation

cephalosporins are almost exclusively directed towards the treatment of human diseases, such as skin and tissue infections, pneumonia, pelvic inflammatory disease and other infections. Only two cephalosporins are currently approved by the Food and Drug Administration for use in food-producing animals – ceftiofur and cephapirin (FDA, 2012). These two antibiotics are only used for therapeutic purposes in individual animals. Due to their high efficacy in treating human diseases, no cephalosporins have been approved for growth promotion in herds or flocks of food-producing animals (FDA, 2012). However, beta-lactam antibiotics, including cephalosporins, are the most widely used antimicrobials in the world.

There has been strong selective pressure, which drives diversification and rapid spread of beta-lactam resistance not only in clinical settings, but also in the environment (Galan *et.al*, 2013). For instance, in multiple Chinese cities, samples obtained from sewage yield cephalosporin concentrations as high as those required to select for organisms that contain extended-spectrum beta-lactamase enzymes (Baquero *et.al,* 2008). Additionally, high resistance to third- and fourth-generation cephalosporins has been found in six Chinese rivers with significant human interactions, where people use the water for personal and agricultural needs (Chen *et.al*, 2012).

Another important aspect of this study is the resistance found to ampicillin, piperacillin and piperacillin/tazobactam. These three antibiotics belong to the penicillin family, a large class of beta-lactam antimicrobial agents. All three are of critical importance to human infections caused by *Enterococcus, Listeria* and multiple multidrug resistant *Pseudomonas* strains (Collignon *et.al.*, 2009). A study has demonstrated that the potential route of transmission of piperacillin and piperacillin/tazobactam resistance from nonhuman sources to humans happens through *Enterococcus* species, *Enterobacteriaceae* organisms (i.e. *E.coli*) and/or *Pseudomonas*

aeruginosa (Collignon *et.al*, 2009). Considering that neither of the aforementioned antibiotics is approved for use in livestock, the resistance found in plasmids captured at Muddy Creek is not only of interest for further investigation, but also of great public health concern.

Ciprofloxacin is an antimicrobial agent from the quinolone family. It has a critical importance to human medicine due to its ability to treat a wide spectrum of infections: *E. coli*-caused urinary tract infections to infections caused by the more invasive *Campylobacter, Salmonella* and multidrug resistant *Shigella* organisms (Collignon *et.al.,* 2009). Ciprofloxacin is approved for human use only (Idowu *et.al*, 2010), which is why the presence of decreased susceptibility to ciprofloxacin in the BL3 plasmid was a surprising find. On the other hand, enrofloxacin, an antibiotic from the fluoroquinolone family, is used exclusively in animals (Idowu *et.al*, 2010). McDermott's lab from the University of Maryland has found that resistance to ciprofloxacin can be developed in poultry treated with enrofloxacin due to their proximal natures (McDermott *et.al.*, 2002). Both ciprofloxacin and enrofloxacin inhibit DNA gyrase as their mechanism of action. Therefore, developing cross-resistance between the two antibiotics is plausible (McDermott *et.al*., 2002).

Tetracycline is a broad-spectrum polyketide antibiotic used in both agriculture and human medicine (Chopra, 2001). Unlike clinically-significant antibiotics such as ceftazidime, tetracycline is not critical to human medicine. Nowadays, it is mostly used as acne treatment (Chopra, 2001). However, it is the most widely used antibiotic in livestock (FDA, 2012). As a result, it is not surprising that tetracycline resistance was found in both plasmids in this study – pBL3 and pBL4. The extensive use of tetracycline in agriculture can easily lead to residual antibiotics in manure, which can be spread onto farm lands as fertilizer or excreted directly from

livestock into grazing lands where it subsequently enters into streams and creeks (Baquero *et.al.*, 2008).

Unlike tetracycline, aminoglycosides such as tobramycin and streptomycin fall into the list of critically important antimicrobial agents used in human medicine (World Health Organization, 2013). Their current use is limited to treating enterococcal endocarditis and multidrug-resistant tuberculosis (Collignon, 2009). The route of transmission of tobramycin and streptomycin from non-human to human sources is suggested to be through the *Enterobacteriaceae* family (Hunter *et.al.*, 2010). While streptomycin is an antimicrobial agent approved as a growth promoter, tobramycin is not and is exclusively made for human use only (Collignon *et.al.*, 2009). Considering this fact, a topic for further investigation would be to find how tobramycin resistance genes have made their way into Muddy creek.

Besides cross-resistance, which was discussed earlier in this section, there have been other scientific ideas as to how resistance genes associate with each other and move together to, from, and within different bacterial communities. The Selfish Operon Model is one model demonstrating the transfer of antibiotic resistance (Lawrence, 2000). Besides the classical way of chromosomal material exchange, Lawrence and his team also describe exchange via mobile genetic elements. For instance, organisms encoding resistance to more than one antibiotic have higher potential fitness than those encoding resistance to only one antimicrobial agent hence the ability of the former organisms to survive in more and different environments (Lawrence, 2000). Genetic linkage, also referred to as "hitchhiking", where multiple antibiotic resistance genes are linked in tandem arrays on elements such as integrons, plasmids and transposons, means that selection for any one of the resistance genes may result in selection for the entire element (Summers, 2002). Resistance genes carried simultaneously on plasmids, together with

Lawrence's "multi-resistance encoding plasmids increase organism's overall fitness" hypothesis, give a reasonable explanation to the multi-resistivity found on plasmids pBL3 and pBL4 of this study.

Initially, only ceftazidime and cefotaxime were selected as antibiotics for the exogenous plasmid capture. However, the results from the first exogenous capture showed growth of unknown bacteria on the combination antibiotic plates (namely – rif/cef and rif/caz). The contaminant source is yet to be determined since all procedures were executed in a sterile, laminar flow hood following precise aseptic technique. One possible explanation to this occurrence is human error. Another possible reason is wrong concentration dosage of the antibiotics on the antibiotic-amended TSA plates. In this study, transconjugants not verified as conjugated recipient *E.coli* stain LA61 were excluded from further investigation. Another issue, encountered during processing the sediment samples, was with the plasmid preparation gels. Originally, it was hard to visualize the results from the BOX rep-PCR due to incorrect voltage and staining reagents. Consequently, it was established that the optimal running voltage was 90V for time approximately 1 hour and 45 minutes. The staining reagents initially used were replaced with more concentrated ethidium bromide for better visualization outcome.

Future work on this research project could include the use of native stream organisms (such as *Pseudomonas* or *Aeromonas*) as capture strains instead of using a laboratory *E. coli* LA61 strain. *Aeromonas*is of interest because it has recently been found that the organism simultaneously harbors multiple resistance genes in wild-growing Mediterranean mussel (Maravic, 2013). An interesting topic of investigation would be to see how many different resistance genes are harbored in fish found in streams around Harrisonburg, VA. In addition, investigating *Pseudomonas* species as potential recipients of exogenously isolated plasmids

would give an insight into the hypothesis that streams serve as reservoirs for resistance genes because *Pseudomonas* is found freely in nature (Pirnay, 2005). Specifically, *Pseudomonas aeruginosa*, as an opportunistic pathogen, is dangerous to humans in both the environment and in clinical settings. The species is known to grow and thrive in diverse environments – from water-based to terrestrial – which makes it a good candidate for investigation (Pirnay, 2005). Another reason why *P .aeruginosa* should be investigated is because it has been found that the species has the potential to contain more than 120 beta-lactamase enzymes, which suggests that it may play an important role in the transfer of beta-lactam resistance genes from the environments to other bacteria and even to humans (Galan, 2013).

In conclusion, this study looked for transmissible plasmids conferring resistance to beta-lactam antibiotics in stream sediment. It resulted in the finding of two plasmids which encoded resistance to a variety of antibiotics, including agents exclusively reserved for clinical use only. While the specific donor(s) of the plasmids is/are unknown, it is obvious that the stream sediment harbors transmissible plasmids that encode simultaneous resistance to multiple antibiotics. What is more, resistance to antibiotics not approved for use in livestock was found in the course of the study. Currently, the diversity of the determined resistance supports the hypothesis of a resistance gene reservoir found on transmissible plasmids and located in an agriculturally-impacted stream. Even though some antibiotics are approved for use in food-producing animals, many of the antibiotics found in this study are not meant to be in the environment. This major finding raises the question of how the use of such clinically-significant antibiotics may impact natural bacterial populations and more importantly, how we will manage the threat of a constantly growing reservoir of resistance in environmental bacteria.

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