Detection of Antibiotic Resistance Genes (ARGs) in Freshwater Streams of West Central Georgia

Jacob Harman & Molli Newman
Faculty Sponsor: Dr. Molli Newman
Department: Biology

Abstract

Antibiotic resistance is becoming one of the most critical health crises of the coming century (Pei, et al., 2006). These resistant microbes come about through a genetic roulette that occurs when antibiotics are introduced into the environment through routes including wastewater and industry discharge and results in increased concentrations of resistance genes (Larsson, 2014; Barancheshme and Munir, 2018). In this study, the presence or absence of tetracycline, sulfonamide, and aminoglycoside antibiotic resistance genes were tested for in freshwater stream sediment.samples. This screening was conducted using PCR-based detection methods. Due to the universal nature of the tetracycline primer pair that tested for multiple resistance genes, it was expected that tetracycline resistance would be the most frequently detected. Sampling sites were chosen with regards to relative human activity and land usage. Sites with the highest level of human activity were predicted to have the highest number of positive results for resistance genes. DNA was extracted from all sediment samples, and PCR amplification was performed according to primer literature specifications in order to determine the presence or absence of a particular antibiotic resistance gene class. PCR products were verified using gel electrophoresis and visualized with ethidium bromide on a LiCor imaging device. Three out of the four sampling sites were positive for sulfonamide resistant genes. No sites tested positive for tetracycline or neomycin (aminoglycoside) resistance. The sulfonamide resistance that was found is thought to have been introduced through agricultural practices in agricultural areas and through wastewater in urban areas. The lack of tetracycline and neomycin resistance was thought to be the result of attenuation and periodical presence in stream waters.

Introduction

Since the discovery of penicillin by Alexander Fleming in 1928, antibiotics have become a central part of the medical profession. They are prized by physicians due to their ability to stop the growth and spread of bacterial infections. The problem that comes with antibiotic use is that microorganisms are dynamic in their ability to react to an environment. While the majority of a colony may die when the environment changes, there is always a chance that one or more cells contain a mutation that allows them to survive in the new environment and in turn produce colonies that can survive in the new conditions. By this mechanism, antibiotic resistance arises (Barancheshme and Munir, 2018).

In 2017, the Centers for Disease Control (CDC) reported that 30% of antibiotic prescriptions were unnecessary (CDC, 2017). By prescribing unnecessary antibiotics, resistant microbes are given a selective advantage. This reaction has led to an increase in the development of resistance genes and the microorganisms that can use these genes (Hu at al., 2008). The lack of effectiveness in treating bacterial infections through antibiotics has had a pronounced effect on mortality rates. Annual deaths associated with antibiotic resistant microbes have been estimated to be 700,000 worldwide (Padivara, et al., 2018). This increased use of antibiotics and the development of resistance has led to both antibiotics and resistant microbes being found in aquatic ecosystems. A 2018 literature review of wastewater treatment research found that wastewater plants were unable to completely remove many antibiotics before they were introduced into the environment. They also stated that this introduction of antibiotics into the aquatic environment has caused bacteria to develop resistance at a very high rate (Barancheshme and Munir, 2018). This increase in antibiotics and their resistance genes in aquatic ecosystems makes them an emerging pollutant of concern.

In this study, the presence or absence of antibiotic resistance genes (ARGs) in freshwater streams of varying surrounding land uses was determined. Tetracycline, sulfonamide, and aminoglycoside ARG classes were targeted due to their broad-spectrum actions and their use in healthcare. This study hypothesized that the presence of ARGs in freshwater stream sediment would be increased in streams experiencing more urban land use and higher human activity in the same manner that was observed in similar studies (Pei, et al. 2006).

Materials and Methods

Study sites and sampling

Sampling sites were chosen similar to those chosen in Pei et al. (2006) taking into account both surrounding land use and human activity levels. Both land use and human activity levels of a given site were determined by observations made about the surrounding land and general human activity that could be observed at each sampling site (e.g. discarded materials, roads, bridges, etc.) as well as accounting for upstream inputs. Four freshwater streams were chosen (Figure 1). Sampling methods were adapted from Pei, et al. (2006). Samples were taken in a cross-sectional manner from both banks and midstream using a sterile, inverted petri dish. The sediment samples for each site were stored in a Whirlpak bag and homogenized. All samples were kept cool until they could be returned to the lab and were then stored frozen (-20°C) until DNA extractions could be performed.

Study Site	Land Use	Human Activity Level
Tanyard Creek (TC)	Urban	High
Park Creek (PC)	Urban	Medium
Long Cane Creek (LCC)	Forested	Low
Harman Creek (HC)	Agricultural	Low

Figure 1. Description of sampling sites (A. Long Cane Creek, B. Park Creek, C. Harman Creek, D. Tanyard Creek)

DNA Extractions

The MoBio Power Soil DNA Isolation kit and accompanying procedure was used to extract DNA from sediment samples (MoBio Lab, United States). Approximately 0.25 grams of sediment was weighed out for use in extraction, and a final volume of 100 µL was used for DNA elution. DNA extracts were then stored at -20°C until use in PCR. DNA concentration was determined using a Nanodrop, and average DNA concentrations are presented in Table 1.

Table 1. Average DNA concentration per sample for each site.

Study Site	**DNA Concentration (ng/µL)**
Long Cane Creek	10.44
Park Creek	2.48
Harman Creek	2.76
Tanyard Creek	0.92

PCR Amplification

PCR amplification was performed according to primer source literature (Table 2). Nuclease-free water was used as a template for negative controls, and a colony of phenotypically-resistant *Pseudomonas aeruginosa* was used as a positive control.

The tetracycline primer pair (Ribo2) used in this study was a degenerative primer pair that tested for multiple tetracycline resistance genes (Aminov, et al, 2001). The Ribo2 primer pair tested for *tetB*, *tetM*, *tetO*, *tetQ*, *tetS*, *tetT*, and *tetW* genes which encode for ribosomal protection proteins that prevent the binding of tetracycline antibiotic to bacterial ribosomes (Roberts, 1996). A total reaction volume of 25µL was used for the Ribo2 primer pair. The reactions contained 12.5 µL (1X) GoGreen Master Mix, 2.5 µL of each 5 µM primer, 2.5 µL nuclease free water, and 5 µL of template DNA. Samples were then placed into thermocycler and amplified according to specifications of Aminov, et al. (2001). The program included an initial denaturation at 94°C for 5 min, 22 cycles of denaturation at 94 °C for 5 minutes, annealing for 30 seconds with 1°C decrements at temperatures of 72°C to 50 °C, and extensions at 72°C for 30 seconds; 20 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds; and a final extension at 72°C for 7 minutes. After PCR amplification was completed, the samples were then stored at -20°C until samples could be visualized.

Table 2. Primers used in amplification procedures and references.

Antibiotic Resistance Class	**Primer Target**	**Amplicon Size (bp)**	**Reference**
Tetracycline	Ribo2 (universal)	1,187	Aminov, et al.,2001
Sulfonamide	sul(I)	163	Pei, 2006
	sul(II)	191	Pei,2006
Aminoglycosides	npt(II)	206	Zhu, 2007

Sulfonamide primer pairs (sul(I) and sul(II)) were traditional primer pairs used to detect sul(I) and sul(II) resistance genes that encode for mutations in key antibiotic targeted enzymes that can allow the enzymes to continue functioning in the presence of antibiotics (Triglia, et al.,1997). Sul(I) and sul(II) primers were adapted from Pei, (2006). This article states that 20 ng of DNA template was necessary for amplification. Sul(I) and sul(II) primer pairs used a total PCR volume of 25 µL. Each reaction contained 1X GoGreen Master Mix, 0.2 µM primers, 20 ng of template DNA, and the remaining volume of nuclease free water. Samples were then amplified using a thermocycler according to specifications of Pei, 2006. The thermocycler was set to initial denaturing at 95°C, 50 cycles of 15 seconds at 95°C; 30 seconds at annealing temperature (65°C sul(I), 57.7 °C sul (II)); and 30 seconds at 72°C. After PCR amplification was completed, the samples were stored at -20°C until samples could be visualized.

Npt(II) was a traditional primer pair encoding for neomycin phosphotransferase, an enzyme that can act on neomycin (an aminoglycoside) and change its structure so that it is no longer functional (Yenofsky, et al., 1990). Npt(II) primers used in this study were adapted from Zhu, 2007. This article stated that PCR amplification with npt(I I) primer required 4 µL of DNA sample but gave no information on DNA concentration needed (Zhu, 2007). Npt(II) PCR reactions used a total reaction volume of 20 µL. Each reaction contained 1X GoGreen Master mix, 0.1 µM primers, 4 µL of template, and the remainder volume nuclease free water. Samples were amplified using a thermocycler according to specifications of Zhu, 2007. Thermalcycler conditions were as follows: 1 cycle of 95°C for 4 minutes; 30 cycles of 95°C for 30 seconds, 55°C for 45 seconds, and 72°C for 1 minute, with a final cycle of 72°C for 7 minutes. After PCR amplification was completed, the samples were stored at -20°C until the results could be visualized.

PCR products were verified using gel electrophoresis with ethidium bromide staining. Standard 1% agarose gels and (1X) TBE buffer were used. Five microliters of each reaction were added into wells along with 5 µL of a 100bp ladder. After sample loading, the gel was submitted to 120 volts for 30 minutes. The resulting migration patterns were imaged using LiCor gel imager (Figure 2). Two replicates were verified per sample.

Results

Images of PCR products are presented in Figure 2. Positive results were noted in the 163-bp regions of the sul(I) primer gel for Harman Creek and Tanyard Creek. Positive results were noted in the 193-bp region of the sul(II) gel for Park Creek, Harman Creek, and Tanyard Creek. No Ribo2 or npt(II) amplification was noted at any site.

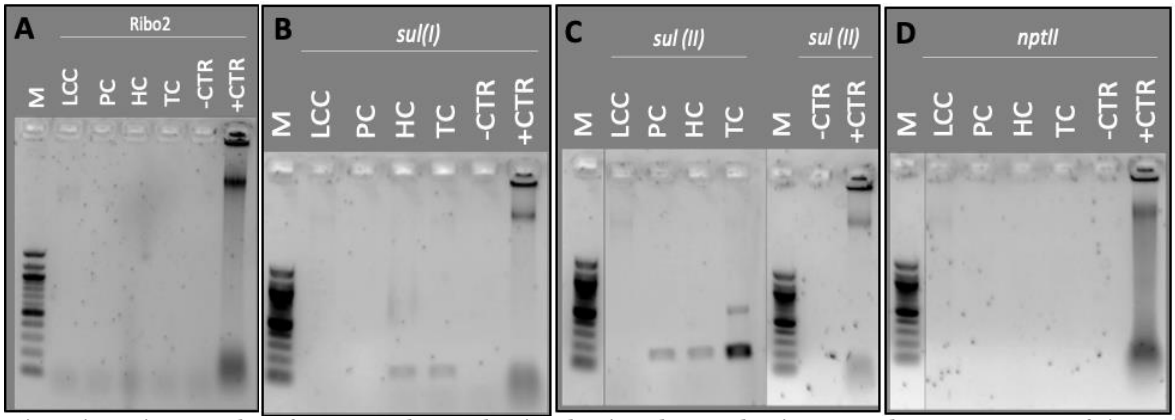

Figure 2. LiCor imaging results of PCR products obtained using denoted primers and DNA extracts of site sediment. M denotes a 100-bp ladder (New England Biolabs), LCC denotes Long Cane Creek, PC denotes Park Creek, HC denotes Harman Creek, TC denotes Tanyard Creek, -CTR denotes negative control. +CTR denotes positive control.

Discussion/Conclusions

Overall, three of the four sampling sites were positive for sulfonamide resistance genes, and no tetracycline or neomycin resistance was detected. Sulfonamide resistance being found in areas with differing human activity and differing land use would suggest that presence of this resistance is correlated to both higher human activity levels and presence of urban and agricultural land usages. This finding is similar to the results that were found in Pei, (2006). The finding of sulfonamide resistance in areas of low human activity and high agricultural land use correlate to Hou et al. (2014) that found sulfonamide resistance accounted for 70% of the resistance found in samplings of livestock feedlots in Northern China. This article detected sul(I) genes at all sample sites tested (including pristine, agricultural, and urban sites) and

sul(II) at a site that had increased levels of urban and agricultural activity. These results are supported by Luo, et al., (2014) that showed sulfonamides to be significantly abundant in sediment samples when compared to water samples. This would increase the potential for resistance that would be needed by microorganisms to survive in the sediment of these sites.

The lack of tetracycline and neomycin resistance amplification suggests that, in the nature of this study, resistance to these antibiotic classes is not correlated to human activity or land usage. These results are similar to the results of Pei, (2006) that found some resistance genes in all sites, but other resistance genes which were found at only one site. Luo et al., (2011) performed an analysis in the Haihe River Basin, China and found that tetracyclines were most prone to attenuation (degradation by adsorption, dilution, photolysis, hydrolysis, and biodegradation) and resulted in tetracycline detection decreasing as distance to introduction site increased. The researchers also found that tetracycline had a high pseudo-partitioning coefficient meaning that tetracyclines are able to penetrate into sediment readily rather than stay in water. This was also supported in Huo et al, 2014 who found that tetracyclines were more prone to sorption and retention in the soil rather than in water. This would mean that as the distance between the introduction site increases, the level of detectable antibiotics decreases. In the event that antibiotic-resistant microbes are introduced in the same site, they would be selected for, but as tetracycline enters the sediment and is no longer carried downstream, the resistant microbes would no longer have an advantage and could be outcompeted further downstream. This would result in a decrease in the amount of detectable genes as the distance from the introduction site is increased. The lack of neomycin-resistance genes could be explained by the timing of site sampling. Zhu, 2007 reported in a longitudinal study that took multiple water samples over a course of 24 months that nptII did not have a consistent presence and was most likely being introduced and then outcompeted. This would explain the lack of nptII amplification in the current study.

In summary, the results of this experiment suggest that sulfonamide resistance genes are influenced by the presence or lack of urban and agricultural activity and levels of human activity and correlate to the results that have been found in other studies. The nature of tetracycline and aminoglycoside resistance gene abundance in relation to human activity levels and land usage cannot be determined from the data that has been gathered from this experiment. This data is supported by other studies that have shown antibiotic resistance genes to be a pollutant of concern and supports the conclusion that this is a widespread problem rather than a area of specific concern.

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Citations

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