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Presence of antibiotic resistance genes from wastewater treatment plant effluent in Northwest Arkansas

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ABSTRACT

Antibiotic resistance genes (ARGs) among bacterial populations are causing increasing concern with medical and agricultural implications. While the effluent of wastewater treatment plants (WWTPs) is treated with a variety of antimicrobial methods, bacteria and the genetic material that is able to pass on antibiotic resistance to environmental populations are not completely destroyed. Ampicillin (amp), tetracycline (tet), and sulfonamide (sul) antibiotics have been detected in Northwest Arkansas (NWA) streams, and IncP plasmids—which are especially notorious for containing antibiotic resistance genes and have been detected after disinfection in NWA WWTPs—are known to carry ARGs for those antibiotics. The objective of this inquiry was to determine whether ARGs of commonly used antibiotics (*ampC* and *oxa2* for ampicillin, *tetA* for tetracycline, and *sul1* for sulfonamide resistance) were present in effluent following disinfection that demonstrated variable reduction in IncP plasmid numbers. DNA was extracted from water collected from a WWTP that uses ultraviolet light and a WWTP that uses chlorination and was participating in a pilot-scale ozonation test. Three of the four ARGs were detected using polymerase chain reaction (PCR) both before and after all three disinfection methods. The ampicillin resistance gene *ampC* was the only gene that was detected in less than two-thirds replications either before or after disinfection. Given the PCR results and previous quantitative PCR analysis of IncP plasmid concentrations, it appears there is little reduction of ARGs after disinfection. These data are important in understanding the role of WWTPs in contributing to the spread of antibiotic resistance in the environment.

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MEET THE STUDENT-AUTHOR

Ryan MacLeod

I am a proud Arkansan, and was born and raised in Little Rock. I graduated from Little Rock Central High School in 2010. The following fall semester, I began studying Biology at the University of Arkansas with a minor study in Environmental, Soil, and Water Science. I approached Dr. Mary Savin about research opportunities in her lab during my sophomore year, and she graciously allowed me to conduct my research study in her lab and provided me with mentoring and advising throughout the project. I received funding from the State Undergraduate Research Fellowship and the Honors College to facilitate my research endeavors.

I plan to attend medical school at the University of Arkansas for Medical Sciences where I am enrolling in a dual MD/PhD program in order to practice medicine and continue fulfilling my research goals. Ultimately, I hope to manage my own research programs in an academic hospital.

INTRODUCTION

Since the discovery of antibiotics and their medical, agricultural, and industrial uses, widespread use of antibiotics has contributed to antibiotic resistance in clinical and natural settings (Karlowsky et al., 2003, Wenzel et al., 2003). As antibiotic resistance spreads throughout bacterial species, many pathogenic bacterial infections are becoming increasingly difficult to treat in humans and agricultural animals (Aminov and Mackie, 2007). Within the last ten years, there has been worldwide recognition of the increase in rates of antimicrobial resistance, and an increase in the frequency of species with multidrug-resistance in a clinical setting has been demonstrated (Cantón, 2009).

One area of particular concern is the dissemination of broad-host range (BHR) plasmids containing antibiotic resistance genes (ARGs) throughout bacterial populations in the environment. Some examples of BHR plasmids are the IncP, IncN, and IncQ families of plasmids. They carry a wide range of resistances to compounds including antibiotics (β-lactams, tetracylines, sulfonamides, erythromycins, and others) and heavy metals (Schlüter et al., 2007). Broad-host range plasmids are able to transfer to and replicate within many different species of bacteria. Even when BHR plasmids themselves do not contain ARGs, they may still mobilize plasmids that do encode resistance yet have no mechanism to transfer themselves by facilitating the formation of a conjugation bridge and recruiting DNA polymerase (Davison, 1999; Thomas and Nielsen, 2005).

The IncP plasmid is a BHR plasmid that is known to carry antibiotic resistance genes (Schlüter et al., 2003). It is considered to be the most promiscuous of the BHR plasmids that have been studied. The IncP plasmids are also known to encode heavy metal—such as mercury—and chlorinated organic compound resistance (Schlüter et al., 2007). These plasmids are found worldwide and have been detected in enteric bacterial species that had not previously contained IncP plasmids before widespread use of antibiotics (Smith and Thomas, 1987).

The IncP plasmid family is commonly detected in wastewater treatment plants (Dröge et al., 2000). Wastewater treatment plants (WWTPs) are considered hotbeds for ARGs and for plasmids that are capable of the horizontal transfer of these genes across species of gram-negative bacteria, even reaching gram-positive species and eukaryotic species such as yeast (Schlüter et al., 2007; Dröge et al., 2000). These WWTPs are seen as interfaces between different environmental compartments including hospitals, surface waters, and residential wastes. The aerators and clarifiers of WWTPs have high bacterial densities and conditions that promote metabolic activities that facilitate genetic exchange in the treatment facil-

ity (Mancini et al., 1987). Among the mobile genetic materials that can transfer resistance genes, several are BHR plasmids (Schlüter et al., 2007). Although the effluent of WWTPs is treated with a variety of antimicrobial methods, none of the current methods completely destroys all the bacteria or mobile genetic material capable of passing on antibiotic resistance (Asfahl and Savin, 2012).

Asfahl and Savin (2012) detected a 95% reduction in IncP plasmids using an experimental ozonation disinfection procedure. Despite a significant reduction in plasmid copy number, 2.2×10^5 copies per liter of the IncP gene remained in effluent to be discharged into the stream. Any amount of these plasmids that is released into the environment can result in the replication and dissemination of antibiotic resistance genes when they enter bacterial populations. Examples of common antibiotics—some of which are found in Northwest Arkansas (NWA) streams and all of which have resistance genes present on IncP plasmids—are ampicillin, tetracyclines, and sulfonamides (Galloway et al., 2005; Haggard and Bartsch, 2009; Schlüter et al., 2003).

The goal of this research is to determine whether resistance genes of commonly used antibiotics are present in effluent known to contain and demonstrate varying levels of reduction in IncP plasmids following disinfection. Previous research evaluated reduction in IncP plasmid number in effluent as a known carrier of resistance determinants; however, destruction of antibiotic resistance genes was not measured directly and needs to be confirmed or refuted. The objective of this experiment was to determine the presence of these resistance genes in the samples both before and after disinfection by chlorination, ozonation, and UV radiation.

Based on the amount of DNA left after disinfection in the effluent of the WWTPs and the concentration of resistance genes on IncP plasmids (Asfahl and Savin, 2012), genes that code for resistance to the antibiotics mentioned above—ampicillin, tetracyclines, and sulfonamides—were expected to be found. Since ampicillin and tetracycline genes were detected more frequently in WWTPs (Yang et al., 2012; Liu et al., 2012), those resistance genes were expected to be more prevalent in the samples than the sulfonamide resistance genes.

MATERIALS AND METHODS

The DNA used in this experiment was extracted using a bead-beating protocol from water samples before and after three disinfection techniques (ultraviolet light in Fayetteville, chlorination in Springdale, and ozonation pilot study in Springdale) in NWA WWTPs (Asfahl and Savin, 2012). Single one-liter samples were taken three different days from the Fayetteville West WWTP and six different days from the Springdale WWTP. There were three replications for Fayetteville's upstream and ultraviolet-treated samples, three replications for Springdale's ozone-treated samples, and six replications for Springdale's upstream and chlorine-treated samples. The DNA was cleaned by ethanol precipitation, quantified by Nanodrop spectrophotometry and has been maintained in a -77 °C freezer. Polymerase chain reaction (PCR) was used to determine the presence of the resistance genes *ampC* and *oxa2* (resistance to ampicillin), *tetA* (resistance to tetracyclines), and *sul1* (resistance to sulfanomides). Template DNA was amplified in 20-µL volumes in a PTC-200 thermocycler (MJ Research, Waltham, Mass.) in the presence of 1X PCR buffer,1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (Promega Corporation, Madison, Wis.), appropriate primer (Table 1), 0.04 % bovine serum albumin (Merck KGaA, Darmstadt, Germany), and GoTaq DNA polymerase (Promega, Madison, Wis.).

Positive and negative controls were run with every batch of PCR reactions. A plasmid (RP4) in the *IncP* family was used as the positive control for the *tetA*, *oxa2*, and *sul1* genes, while an environmental sample that was found to consistently amplify the correct fragment size was used for the *ampC* gene. The negative controls consisted of all the reagents used in the PCR batch without the addition of template DNA. Amplification was confirmed using ethidium bromide agarose gel electrophoresis and

Table 1. Polymerase chain reaction primer sequences used successfully with environmental DNA samples.

Gene	Primer Sequence	Annealing Temperature (°C)	Expected Product size (bp) (Source)
ampC	FW- CCT CTT GCT CCA CAT TTG CT	58	189 (Yang et al., 2012)
	RV- ACA ACG TTT GCT GTG TGA CG		
oxa2	FW- TCT TCG CGA TAC TTT TCT CCA	60	177 (Yang et al., 2012)
	RV- ATC GCA CAG GAT CAA AAA CC		
tetA	FW- GCT ACA TCC TGC TTG CCT TC	61	210 (Liu et al., 2012)
	RV- CAT AGA TCG CCG TGA AGA GG		
sul1	FW- CGC ACC GGA AAC ATC GCT GCA C	56	163 (Pei et al., 2006)
	RV- TGA AGT TCC GCC GCA AGG CTC G		

the Kodak EDAS 290 (Eastman Kodak, Rochester, N.Y.). The size of the products was determined using the mass molecular standard ruler (bands from 1000 bp to 100 bp), which also can be used to determine DNA concentrations of the PCR products by comparing the intensity of the bands to the standard's band intensities. Reduction, or the absence of PCR amplification for a particular gene fragment after disinfection, following the presence of PCR amplification upstream of the disinfection procedure was determined to be statistically significant by conducting a T-test.

RESULTS AND DISCUSSION

Three of the four antibiotic resistance genes (*oxa2*, *tetA*, and *sul1*) were amplified across all replications before and after disinfection across all three disinfection protocols utilized in both WWTPs (Table 2, Figs. 1-2). Qualitatively, the intensity of the *sul1* band fragments indicates a concentration of 10-20 ng/μL DNA amplifed in the upstream site (i.e. prior to disinfection) with no reduction of intensity, or DNA concentration amplified, downstream of disinfection (Fig. 1). Similar results were obtained for the *tetA* tetracycline resistance gene fragment (Fig. 2). The *oxa2* gene also amplified in all replications both before and after all three disinfection methods (Table 2).

The fourth antibiotic resistance gene, *ampC*, was only amplified in 33% of upstream replications in the Fayetteville WWTP and 66% of upstream replications in the Springdale WWTP (Table 2). The replications that did contain *ampC* amplification still contained bands after disinfection for the UV-treated Fayetteville effluent, while half of the chlorinated Springdale replications that initially contained *ampC* no longer amplified the gene after disinfection (Table 2). All of the ozonation replications at the Springdale WWTP no longer contained *ampC* amplification after disinfection.

Table 2. Polymerase chain reaction results for presence of antibiotic resistance genes.

WWTP[a]	Number of Replications	Disinfection Method	Amplification (%)			
			ampC[c]	*oxa2*	*tetA*	*sul1*
Fayetteville	3	Upstream[b]	33	100	100	100
	3	UV	33	100	100	100
Springdale	6	Upstream	66	100	100	100
	6	Chlorination	33[d]	100	100	100
	3	Ozonation	0[d]	100	100	100

[a]Wastewater treatment plant.
[b]Upstream samples are for effluent collected prior to disinfection. UV (ultraviolet radiation), chlorination, and ozonation are for effluent collected after disinfection.
[c]The *ampC* and *oxa2* genes code for ampicillin resistance. The *tetA* gene codes for tetracycline resistance and the *sul1* gene codes for resistance to sulfonamides.
[d]These differences by chlorination and ozonation at the Springdale WWTP were significant at $P < 0.05$.

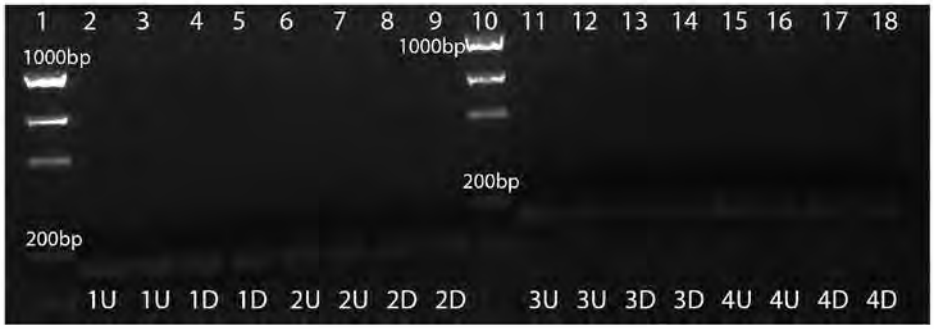

Fig. 1. Selected agarose gel showing polymerase chain reaction products. The gene amplified was *sul1* with an expected product size of 163 bp. Samples from Springdale wastewater treatment plant upstream (U, before) and downstream (D, after) disinfection. Lanes 1, 10 are mass molecular standard. Lanes 2-5 are from replication 1, lanes 6-9 are replication 2, lanes 11-14 are replication 3, and lanes 15-18 are replication 4.

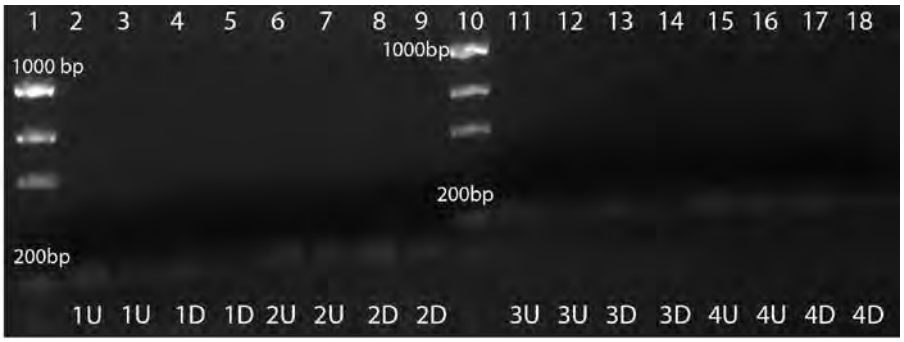

Fig. 2. Selected agarose gel showing *tetA* gene amplification. The expected product size for *tetA* was 210 bp. Samples are from Springdale wastewater treatment plant upstream (U, before) and downstream (D, after) disinfection. Lanes 1, 10 are mass molecular standard. Lanes 2-5 are from replication 1, lanes 6-9 are from replication 2, lanes 11-14 are from replication 3, and lanes 15-18 are from replication 4.

The UV and chlorination treatments of WWTP effluent yielded insignificant reduction levels of IncP plasmid DNA (Asfahl and Savin, 2012). At the Fayetteville WWTP using UV disinfection, 2.7 (± 8.2) × 10^6 copies per liter were detected before disinfection and 0.8 (± 2.2) × 10^6 copies per liter were detected after disinfection (Asfahl and Savin, 2012). Springdale's chlorination disinfection was also not significant with 2.7 (± 1.8) × 10^7 copies per liter detected upstream and 4.2 (± 1.5) × 10^6 copies per liter after disinfection (Asfahl and Savin, 2012). The amplification of *sul1*, *tetA*, and *oxa2* reveal that these were not eliminated from the water samples collected after each of the disinfection methods, and thus may persist and disseminate into new hosts either because of their mobility or facilitated mobility by other genetic elements. These genes are likely to be located either on mobile genetic elements that contain a sequence that facilitates the formation of a junction between two bacteria during the conjugation process or on genetic elements that are replicated through an existing conjugation bridge (Davison, 1999; Thomas and Nielsen, 2005). The IncP plasmids are notorious for both initiating the conjugation process for their own dissemination and facilitating the replication of non self-mobilizing genetic elements (Schlüter et al., 2007). That leaves a possibility for antibiotic resistance genes to be released and spread throughout the environmental populations of bacteria.

The PCR results for *tetA*, *sul1*, *oxa2*, and *ampC* are consistent with the continued detection of total DNA and specifically IncP plasmids upstream and downstream of disinfection (Asfahl and Savin, 2012). From the quantitative PCR results in Asfahl and Savin (2012), it is clear that UV radiation, chlorination, and ozonation do not effectively eliminate mobile genetic elements that carry antibiotic resistance genes from the WWTP effluent. Even following a significant reduction after ozonation in IncP plasmids as indicated by 95% reduction in amplified gene fragment, there were 2.2 (± 0.7) × 10^5 copies per liter remaining. With 45 million liters of water discharged daily from Springdale WWTP, the potential load into Spring Creek is 9.9 × 10^{12} IncP plasmid copies per day. If each IncP plasmid contained one copy of each resistance gene *oxa2*, *sul1*, and *tetA*, then it becomes easier to understand how WWTPs can influence resistance in the environment.

With the exception of the low detection of *ampC*, these results are in agreement with the growing database of antibiotic resistance genes detected in WWTP effluent. Studies involving sulfonamide, tetracycline, and ampicillin resistance genes across a wide range of locations in East Asia and North America have detected many of these genes in the WWTP and associate effluent, specifically *oxa2*, *ampC*, *tetA*, and *sul1* (Liu et al., 2012; Pei et al., 2006; Yang et al., 2012). The *tetA* gene and many other genes conferring tetracycline resistance were detected before and after treatment of antibiotic production wastewater at a manufacturing plant in Hebei Province, China (Liu et al., 2012). The ampicillin resistance gene *ampC* was detected in 100% of WWTPs, and *oxa2* was detected in 93% of the Asian and North American WWTPs tested in a previous study (Yang et al., 2012). The *sul1* gene was detected in sediments of the Poudre River in northern Colorado across all sampling sites, including one downstream from an urban WWTP (Pei et al., 2006).

The results are also consistent with the presence in Northwest Arkansas streams of the antibiotics for which the genes encode resistance. Sulfamethoxazole (part of the sulfonamide group of antibiotics) and, to a lesser extent, tetracycline, have been detected downstream of Fayetteville WWTPs effluent discharge into Mud Creek

(Galloway et al., 2005; Haggard and Bartsch, 2009). The presence of these drugs in the waters can put selective pressure on the environmental populations of bacteria to develop or acquire resistance genes. These genes can be spread in WWTPs, which have been known to have conditions conducive for conjugation, transduction, and transformation (Schlüter et al., 2007; Dröge et al., 2000).

Furthermore, the PCR results showing persistence of antibiotic resistance genes following disinfection are consistent with the linkage of ampicillin, tetracycline, and sulfonamide resistance genes to the IncP plasmid family. The genes *sul1*, *tetA*, and *oxa2* have all been sequenced and linked to multiple plasmids belonging to the IncP family (Schlüter et al., 2007). The absence of the *ampC* gene in many of the replications was unexpected considering the gene's detection in effluent of 100% of WWTPs studied in Asia and North America and the gene's presence on multiple plasmids that carry resistance (Yang et al., 2012). However, these results are interesting since *ampC* is not expected to be associated with IncP plasmids (Yang et al., 2012). The *ampC* gene is located on an assortment of conjugative plasmids including pGC-1 and pGC-2 (Bou et al., 2000). The *oxa2* ampicillin resistance gene is more frequently found on mobile genetic elements than ampC, however, because mobile *ampC* genes are derived from the chromosomal DNA and are not as prevalent on plasmids (Bou et al., 2000).

The nonexistent reduction in the number of samples in which *oxa2*, *sul1*, and *tetA* genes were detected may reflect the results and lack of IncP plasmid DNA destruction detected in Asfahl and Savin (2012) because of the previously stated connection of these genes with IncP plasmids and other DNA. Even though the ozone treatment reduced IncP levels by 95%, it only reduced the total DNA concentrations by 80%. That leaves a possibility for other antibiotic resistance genes that are not associated with IncP plasmids specifically to be released and spread throughout the environmental populations of bacteria.

CONCLUSIONS

Antibiotic resistance gene detection both upstream and downstream of UV irradiation, chlorination, and ozonation treatments of wastewater shows persistence and thus suggests the potential for dissemination of these genes in streams through WWTP effluent. The results from this and similar studies suggest that new methods of disinfection or different policies concerning regulation of DNA concentrations in WWTP effluent need to be developed to avoid the spread of antibiotic resistance genes. The need to at least slow the dissemination of these genes is exemplified by the growing trend of multidrug-resistant, pathogenic species of bacteria. By continuing to allow antibiotic resistance genes to be released into the environment, the reservoir of resistance genes increases and there is a greater chance of pathogenic bacteria acquiring resistance. This creates problems for disease control in clinical, agricultural, and industrial settings. Future research opportunities with this study should involve quantifying antibiotic resistance gene reduction resulting from disinfection using a quantitative PCR analysis.

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