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Kentucky Wastewater Treatment Plants and the Prevalence of Antibiotic Resistance Genes

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Abstract

Antibiotic resistance has been a growing problem in healthcare and agriculture for several decades. Antibiotic resistance has been evolving naturally for millions of years, but the process is also accelerated by human activities. Wastewater treatment plants have been studied as potential sources of antibiotic resistance genes in aquatic environments. In this study, several wastewater treatment plants in southern Kentucky were tested for the presence of blaSHV, blaCTX, and tet(B) using PCR and gel electrophoresis. Preliminary evidence suggests that the wastewater treatment plant at West Hickman may be a source of tet(B) and that the wastewater treatment plant at Silver Creek may be a source of blaSHV.

Introduction

Antibiotics are compounds that are able to inhibit the growth of bacteria. There are many different antibiotics which differ in their effectiveness against different types of bacteria and modes of action in order to work. Some antibiotics have developed naturally over time through the process of evolution, while others have been synthetically produced by humans over the past several decades.

Through the processes of evolution, some bacteria have developed various mechanisms to resist the effects of antibiotic compounds. These traits are genetically coded for by antibiotic resistance genes (ARGs). ARGs may give bacteria resistance to one antibiotic or a class of antibiotics.

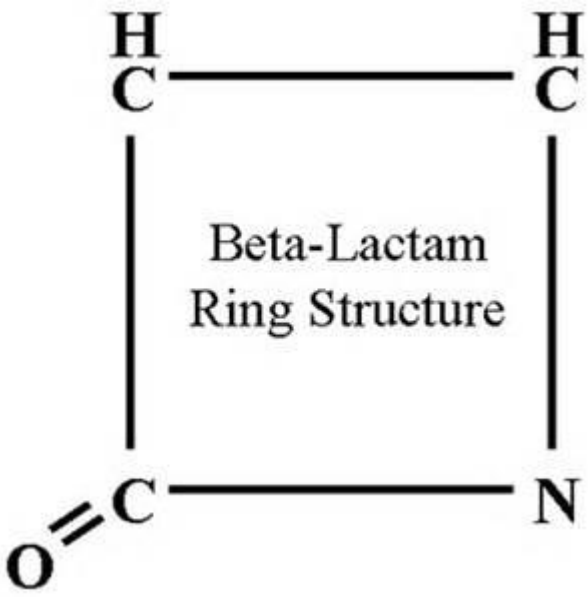
ARGs are passed from one generation to another. Additionally, ARGs can spread from one bacteria to others through several different mechanisms of DNA transfer: transformation, transduction, and conjugation. Transformation occurs when bacteria obtain free DNA from the environment, and transduction occurs when a bacteriophage (a virus that infects bacteria) carries DNA from one bacterial cell to another. Of more interest is bacteria gaining ARGs through the process of conjugation, which occurs when plasmids are transferred from one bacteria cell to another. Plasmids are bacterial DNA that replicate independently of the bacterial chromosome, and some, known as conjugative plasmids, are able to move from one to another.

Tetracycline kills bacteria by inhibiting protein synthesis. It does this by preventing the attachment of aminoacyl-tRNA to the ribosomal accepter (A) site. Tetracyclines are broad spectrum antibiotics and have an absence of major adverse side effects, which has led to their extensive use in treating human and animal infections (1). Dozens of tetracycline resistance genes exist, and there are several different mechanisms through which the genes confer resistance. ARG *tet*(B) confers resistance to bacteria by encoding an efflux pump mechanism (1). Efflux pumps use proteins in order to move compounds across a membrane. The pump encoded for by *tet*(B) exports tetracycline from the inside of the cell to the outside of the cell, thereby lowering the concentration of *tet*(B) inside of the cell (1). The lower concentration of tetracycline inside bacteria protects the ribosomes from being inactivated.

Beta-lactams are a class that share a common structural component, the beta-lactam ring, which gives them their antimicrobial properties. They are often used in order to treat

patients who are infected by Gram-negative bacteria (2). Beta-Lactams are able to kill bacteria by inhibiting the biosynthesis of bacterial cell walls. Specifically, they halt the activity of transpeptidase, an enzyme that is crucial to cell wall biosynthesis in bacteria. The CO-N bond in the beta-lactam ring (Figure 1) acts as a substrate analog to the transpeptidase enzyme (3).

Figure 1 (4): Structure of the beta-lactam ring, a common structural component of beta-lactam antibiotics. The CO-N bond at the bottom of the ring gives beta-lactams their antimicrobial properties



Beta-lactamase ARGs encode the bacterial enzyme beta-lactamase. This enzyme hydrolyzes the beta-lactam ring, making the entire compound inactive. Beta-lactamases are classified into four different groups based on amino acid sequences of the enzyme (3). Additionally, there are two different mechanisms by which beta-lactamases can hydrolyze beta-lactam rings. One mechanism is serine based and the other mechanism requires zinc (3). ARGs blaSHV and blaCTX are in the same group of beta-lactamase resistance genes, ambler molecular class A (5). This class of enzymes is known as penicillinases, and they work using the serine based mechanism (3).

SHV-type extended spectrum beta-lactamases (ESBLs) may be more common in clinical isolates than any other type of ESBL (6), highlighting the importance of the blaSHV gene. Also, SHV-type ESBLs are most often found in *E. coli* and *K. pneumoniae* (7), raising the potential of release near Wastewater Treatment Plants (WWTPs). While it has been hypothesized that SHV genes are part of a mobile unit, it has not been proven (7).

Both blaSHV and blaCTX-M genes have been found on every continent. It is speculated that blaCTX-M type ESBLs are now actually the most common worldwide (5). The CTX-M9 gene is plasmid encoded, and there is evidence suggesting the mobility of many blaCTX-M genes, including blaCTX-M9 specifically (5), meaning these genes can be easily transferred from one bacteria to another.

While blaSHV and blaCTX-M genes share many similarities, there are some differences between the two. For example, blaSHV is more effective against penicillin than blaCTX-M genes are. CTX-M genes exhibit their highest hydrolytic activity against narrow-spectrum cephalosporins. Also, CTX-M genes are enzymatically weak against ceftazidime, marking a distinguishing contrast with SHV enzymes (5).

In this experiment, DNA isolates from samples are tested for the presence of three ARGs: tet(B), blaSHV, and blaCTX-M9. The ARG *tet*(B) is abundant in nature. For example, tet(B) is the most common determinant found in *Enterobacteriaceae* and it provides bacteria that possess it resistance to the antibiotic tetracycline **(8).** The majority of *tet* genes are able to transfer easily from one bacteria to another because they are associated with various types of

mobile units of DNA including mobile plasmids and conjugative transposons (1). This, in part, explains their abundance in nature.

Polymerase chain reaction (PCR) creates many copies of a targeted DNA sequence (Figure 2). First, the sample DNA is combined with primers specific to the sequence that is desired to be copied, free nucleotides, and a special DNA polymerase called Taq Polymerase. Taq Polymerase is unique in that it works at higher temperatures than typical DNA polymerases. Next, the reaction mixture is heated in order to denature the DNA. This makes two single strands of DNA that are now ready for another complimentary strand to be synthesized. Once the DNA is denatured, the primers are synthesized onto each of the single strands by Taq polymerase, which then continues to synthesize the rest of the complementary strands using the free nucleotides. The result is two copies of the targeted DNA sequence (One for each single strand of DNA created by the heating). This process is repeated a number of times, usually 25-30, exponentially increasing the number of targeted DNA sequences with each cycle. Figure 3 shows the steps in one cycle of PCR. Once the reaction is finished, there are millions of copies of the targeted DNA sequence. For this experiment, PCR reactions for specific ARGs were used on isolated DNA samples from WWTPs in order to make many copies of the gene if it was present in the sample.

Figure 2 (9): Diagram showing the exponential amplification resulting from Polymerase Chain Reaction (PCR). The targeted gene is copied, then the original gene and each copy is replicated during each cycle.

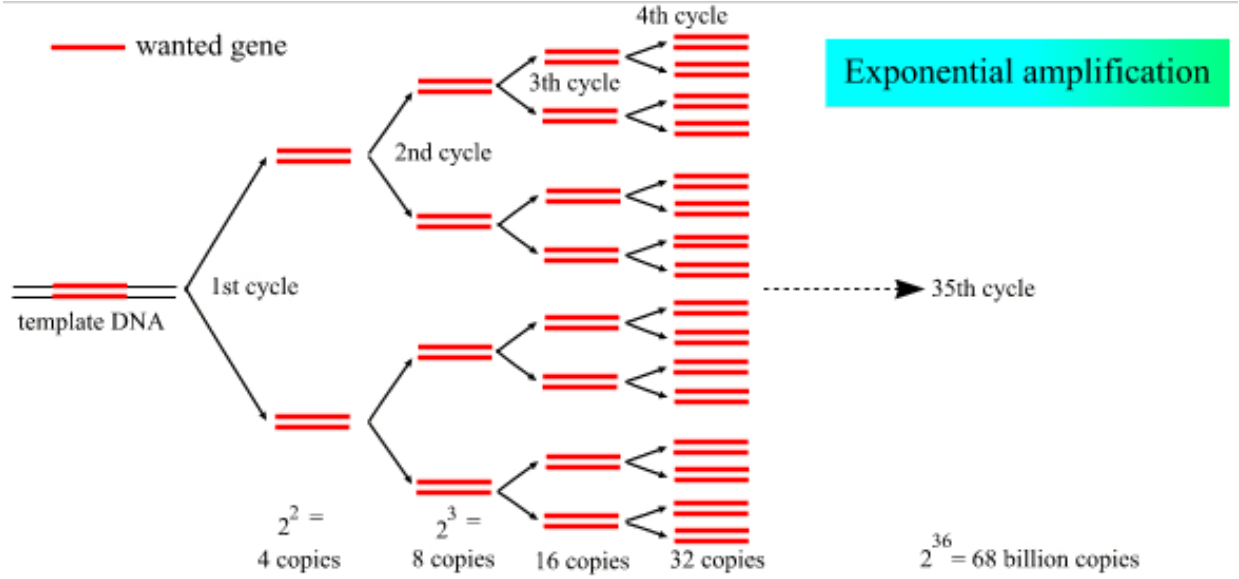
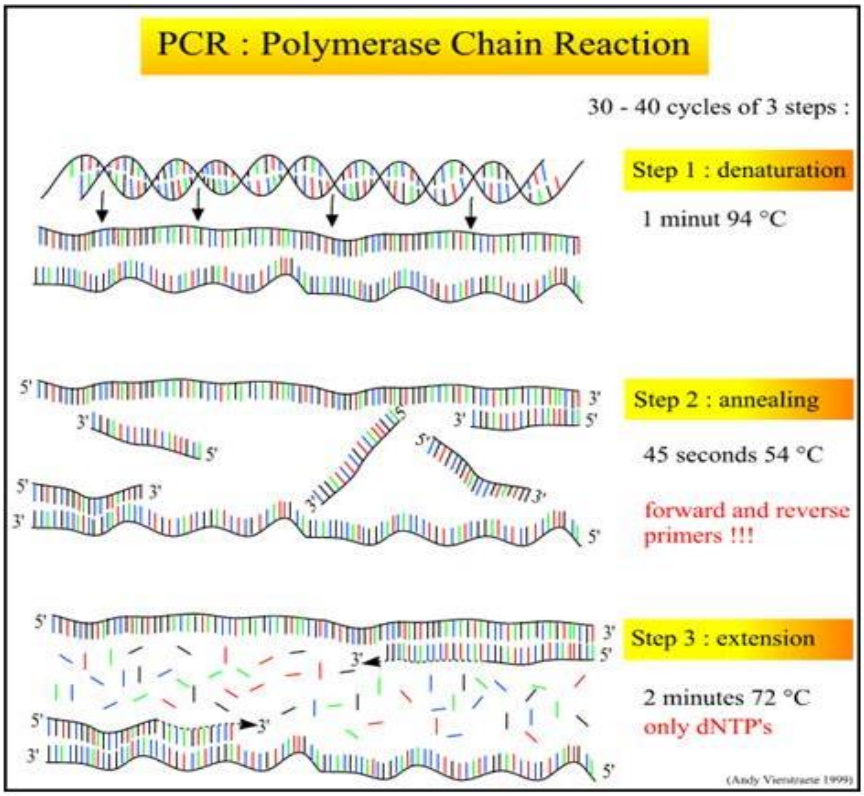
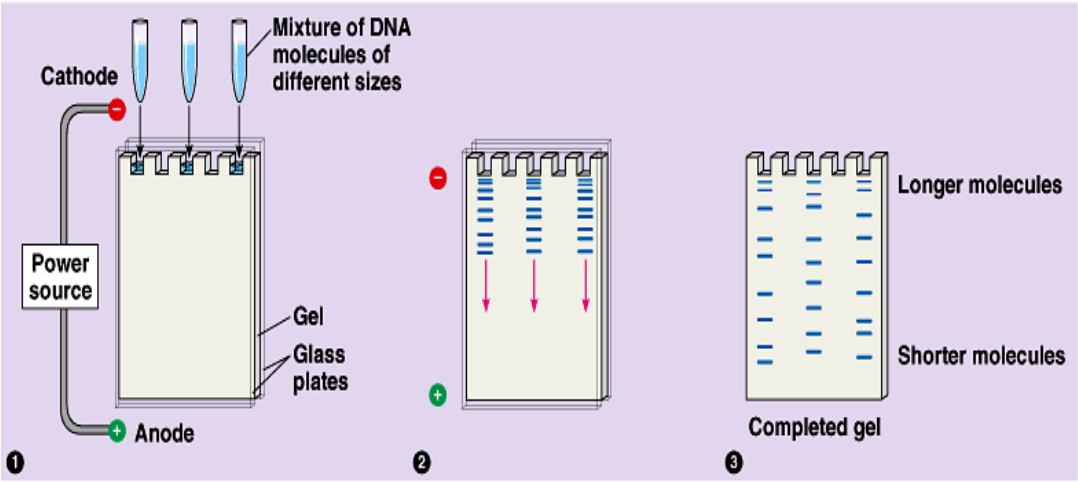


Figure 3 (9): Diagramming the different steps of PCR. During denaturation, the DNA is split into two single strands. During annealing, the primers attach to the targeted gene. During extension, taq polymerase synthesizes the rest of the targeted gene.



Gel electrophoresis (Figure 4) is used in order to visualize the presence of DNA segments of certain lengths. After a PCR reaction is performed, the products are placed into an agarose gel. The gel is connected to a current, with one side being negatively charged and the other side being positively charged. The DNA is loaded into the gel on the negative side. Since DNA is negatively charged, the current will cause the DNA to move along the gel, as it is repelled by the negative charges where it is loaded and attracted to the positive charges on the opposite end. Agarose gel is like a web that does not allow things to pass through it easily. As a result, larger DNA strands will move more slowly through the gel than smaller DNA strands, and relative DNA strand lengths can be compared in a given gel. Also, a DNA ladder, which is a solution of DNA bands of known lengths, is loaded into each gel. This allows for the estimation of the number of base pairs in the bands of DNA seen in the gel. Once the DNA has had sufficient time to move through the gel, the gel is disconnected from the current and examined under ultraviolet (UV) light, where the DNA bands are visible if present.

Figure 4: Depiction of the gel electrophoresis process. The negatively charged DNA experiences electrostatic forces that push it through the gel. The agarose allows shorter molecules to travel faster and farther than larger moleules.



There is already existing research from around the globe that wastewater treatment plants and other human activities are likely causing an increase in the prevalence of antibiotic resistance genes in aquatic environments (11-25). Several factors pertaining to WWTPs may contribute to this increase. Antibiotics in sewage select for ARGs in aquatic environments. Also, several studies have shown that chlorine, a common disinfecting agent in WWTPs, selects for antibiotic resistant bacteria (ARB), though the mechanism by which chlorine-induced antibiotic resistance arises in bacteria is unknown (11). It is also possible that WWTPs create an environment in which resistance genes are more likely to be passed from one bacteria to another (26), which would not only increase the prevalence of ARGs in water but would also increase the likelihood that ARGs become incorporated into the genomes of pathogenic bacteria.

While there is already a large body of evidence that WWTPs are playing a role in increasing the prevalence of antibiotic resistance in aquatic environments, this project is unique in that it is examining a geographic region, south and southeastern Kentucky, that has not yet been studied. This study could shed light on whether or not this phenomenon is happening in SE KY. Additionally, if there is more evidence pertaining to this phenomenon, there is a chance that more attention will be brought to the importance of it, perhaps leading to the idea that this problem ought to be addressed through future research. More data may also bring to light particular methods of water treatment that are more likely to select for ARGs than other methods, which could lead to changes in the way we treat water that could have significant impacts in the fight against antibiotic resistance.

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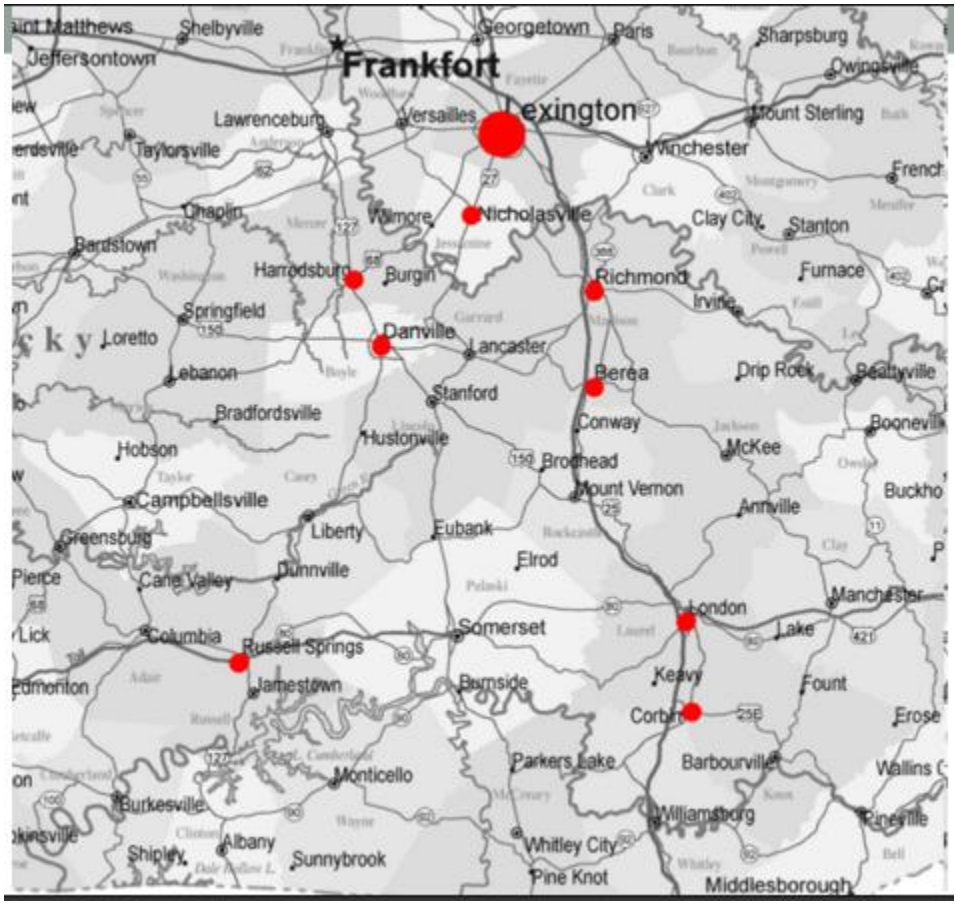
This research builds on previous work of Brooke Johnson (27). Johnson tested for evidence of ARGs *tet*(A), *tet*(B), *tet*(X), and *tet*(O) at several sites (Figure 5), and the research indicated that some sample sites may be increasing the prevalence of *tet*(A) and *tet*(X).

Figure 5: Map of south central KY. Red dots indicate sites of water sources that were sampled.



In order to determine whether or not WWTPs are increasing the prevalence of ARGs, water samples were taken from upstream, effluent, downstream, and from water coming out of each of the WWTPs. If ARGs were found downstream, effluent, or in the water, but they were not found upstream of the WWTP, then it is possible that it is the WWTP that is causing this to happen. If the genes are found upstream in addition to downstream, effluent, or in the

water, then it is likely that the source of the ARG(s) is something other than the WWTP. That source could be natural or some other anthropogenic influence.

One complication of this approach is the fact that many ARGs are naturally occurring without any anthropogenic influences. Bacteria have been evolving for billions of years, since before humans were around to provide selection pressures on bacteria. Even without humans, bacteria have been struggling to survive against natural antibiotics produced by other organisms, and these natural antibiotics have selected for ARGs in bacteria without any human activity involved at all. As a result, there cannot be certainty that, if ARGs are found downstream, effluent, or from the water but not upstream, the WWTP is definitely the cause. It is possible that the ARG found is simply naturally occurring.

The implication and significance of this research is potentially connected to human health and disease. Antibiotic resistance is an increasingly severe problem for health care workers to the point where some bacteria have become so resistant that they are virtually untreatable (28). Given the lack of priority by funding institutions such as the National Institute of Health (NIH) in developing new antibiotics and studying antibiotic resistance (29), it is vital that humanity does what it can to slow the growth and prevalence of resistance genes. The results of this research could give insight into what human activities could potentially be changed in order to slow the process by which resistance genes arise.

Materials and Methods

Each sample was tested for the presence of three antibiotic resistance genes using PCR: tet(B), blaCTX, and blaSHV. The different sets of primers and conditions for PCR are shown in Table 1 (2, 31). Gel electrophoresis was used to separate the PCR products and visualize the presence or absence of the DNA, and the PCR products were stained with gel red and allowed to run on 100 volts for 30-40 minutes.

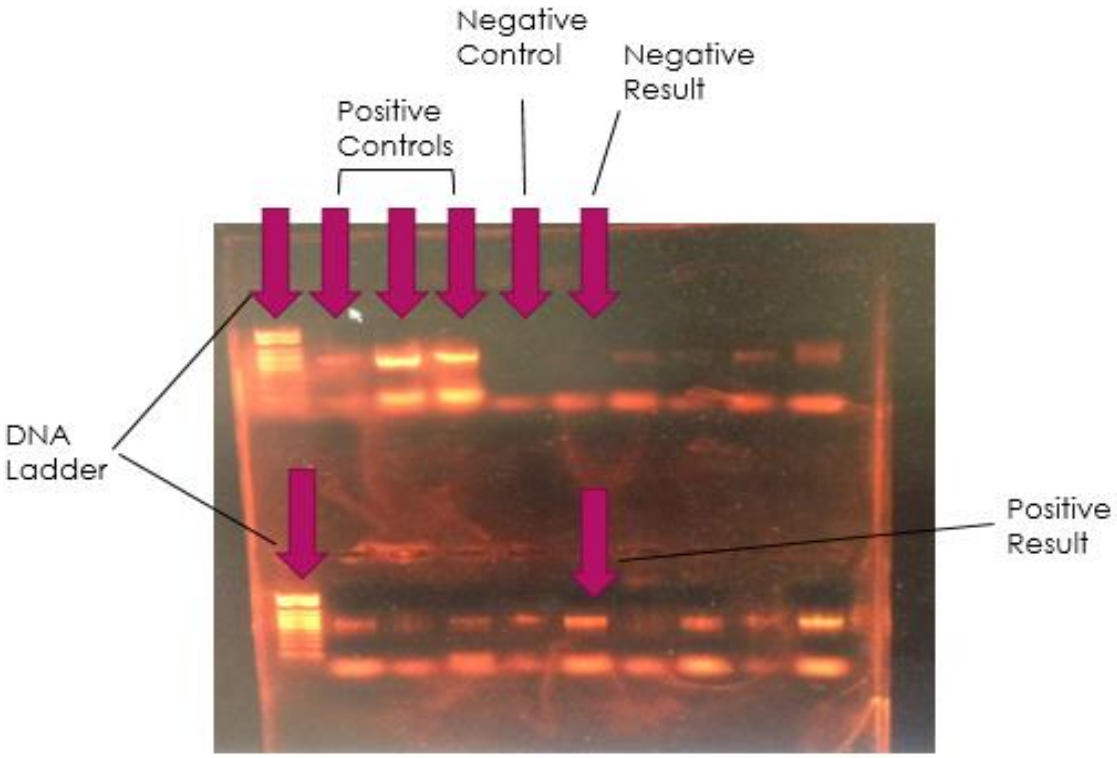
Table 1. PCR Conditions (2, 30)

	Tet(B) Primers 7, 8	BlaCTX-M9 Primers 57, 58	BlaSHV Primers 53, 54	blaSHV Primers 106, 107
Forward Sequence	TTG GTT AGG GGC AAG TTT TG	ATGGTGACAAAGAGAGTGCA	CTTTACTCGCTTTATCG	TTATCTCCCTGTTAGCCACC
Reverse Sequence	GTA ATG GGC CAA TAA CAC CG	CCCTTCGGCGATGATTCTC	TCCCGCAGATAAATCACCA	GATTTGCTGATTTCGCTCGG
Annealing Temp	49^{o}C 59 s	49^{o}C 60 s	46^{o}C 30 s	50^{o}C 60 s
Number of cycles	25?	30	30	30

Results

Tet*(B)* was found in some but not all locations (Figure 6).

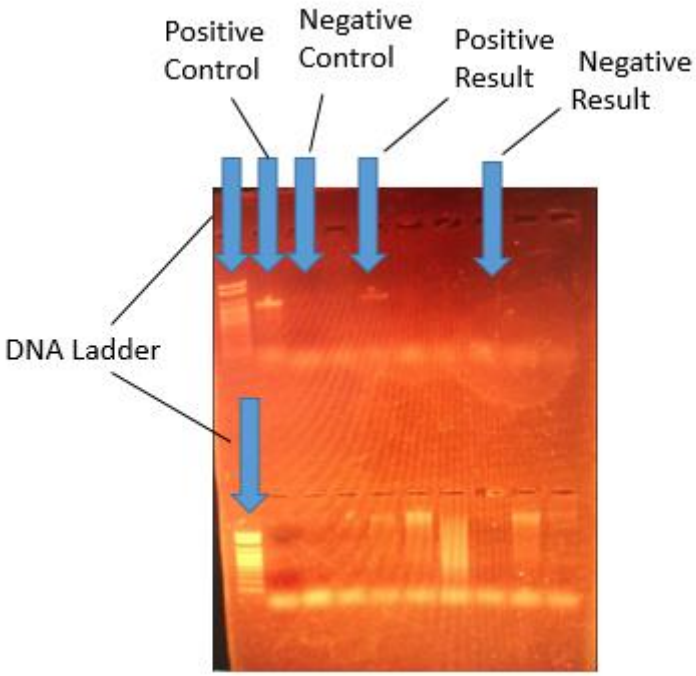
Figure 6. Example of a gel from experiments. Bands at a particular part of the gel indicate evidence of ARGs. The first lane in each row is the DNA ladder. The next three lanes in the 1st row are positive controls, and the fifth lane is the negative control. A negative result can be seen in lane six, and a strong positive result can be seen in lane 16.



The gel has 20 lanes, 2 rows of 10, with DNA or samples loaded into each of them. The first lane on the left of each row is the DNA ladder of a known size. It can be used as a comparison for any bands in the rest of the gel in order to estimate the size of any bands in the gel. The second, third, and fourth lanes on the top row are the positive controls. The next two lanes have no bands at the same length of the positive controls, indicating the absence of ARG *tet(B)* in these samples. The remaining lanes all have bands of varying strengths at the expected length for *tet(B)*, indicating a positive result for the gene. Some bands are very strong, such as the bands in the sixth, eighth, and last lanes of the second row. These would be examples of definite positive results. Other bands, such as the bands in lanes three, five, and seven in the

second row and lane eight of the first row, are not as strong. These bands are inconclusive as to whether they should be considered positive or not. Bands such as these are considered potential positives, meaning any are made with caution.

Figure 7: blaSHV gel. A positive result can be identified in lane 5.



A band is easily seen there at the top half of the ladder. In the fifth lane of the top row, a faint band can be seen at around the same spot as the positive control. This band is evidence of the presence of blaSHV in a sample from water in Silver Creek. The bands visible at the bottom of each row are from primers and do not indicate ARG.

Many samples tested positive for the presence of ARG tet(B). Only one sample, a sample from Silver Creek, tested positive for blaSHV. None of the samples tested positive for blaCTX. Table 2 below summarizes the findings of the experiment.

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Table 2. Evidence of Antibiotic Resistance by Site and Sampling location

Site	Sampling Location	Tet B	blaSHV Primers 53,54	blaCTX	blaSHV Primers 106, 107
Silver Creek	Water	-	-	-	-
	Water	-	+	-	-
	Upstream	?	-	-	-
	Effluent	?	-	-	-
	Downstream	?	-	-	-
Otter Creek (Berea)	Water	?	-	-	-
	Water	-	-	-	-
	Upstream	?	-	-	-
	Effluent	?	-	-	-
	Downstream	?	-	-	-
Danville	Water	-	-	-	-
	Upstream	?	-	-	-
	Downstream	?	-	-	-
Lake Cumberland	Water	+	-	-	-
Pittman Creek	Water	-	-	-	-
	Upstream	-	-	-	-
	Effluent	-	-	-	-
	Downstream	-	-	-	-
Whitley Branch	Water	+	-	-	-
	Upstream	+	-	-	-
	Effluent	+	-	-	-
	Downstream	+	-	-	-
Lynn Camp	Water	-	-	-	-
	Upstream	+	-	-	-
	Effluent	+	-	-	-
	Downstream	+	-	-	-
Town Branch	Water	+	-	-	-
	Upstream	+	-	-	-
	Effluent	?	-	-	-
	Downstream	?	-	-	-
West Hickman	Water	-	-	-	-
	Upstream	-	-	-	-
	Effluent	?	-	-	-
	Downstream	?	-	-	-
Town Creek (Harrodsburg)	Water	?	-	-	-
	Upstream	-	-	-	-
	Effluent	-	-	-	-
	Downstream	-	-	-	-

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Discussion

For a majority of the WWTPs examined, there was no evidence suggesting that they were responsible for the presence of any of the ARGs. None of the samples tested positive for blaCTX. This is somewhat surprising, considering blaCTX is often a prevalent ARG in nature. One sample tested positive for blaSHV. This is also surprising as it too is one of the most prevalent beta lactamase ARGs in nature (2).

The one sample that tested positive for blaSHV came from DNA isolated from the water being released from the WWTP in Silver Creek. Since, the gene was not found upstream of the WWTP, there is reason to believe that the WWTP may be responsible for the presence of the gene. If the effluent and downstream samples had tested positive, there would be more conclusive evidence that this was the case.

A majority of the samples tested were positive for tet(B), indicating that tet(B) is a common naturally occurring ARG or is a common ARG from anthropogenic influences other than WWTPs. While nearly every sampling site tested positive for tet(B) DNA, only two sites supplied evidence that WWTPs were responsible for the presence of tet(B). Other sites that tested positive showed evidence of tet(B) upstream of the WWTP, indicating that the gene was being introduced by some other means.

One of those sites that did suggest a role from WWTPs in causing the presence of tet(B) was Lake Cumberland. Only one sample was taken from Lake Cumberland, and that sample was

taken from the water coming from the WWTP. That sample tested positive for tet(B). Since no samples were taken upstream of the WWTP in Lake Cumberland, it cannot be known whether the gene was also present upstream, which would suggest that perhaps the WWTP was not responsible for the prevalence of tet(B). However, since there is an absence of that evidence, it can reasonably be inferred that the WWTP at Lake Cumberland is potentially releasing tet(B) into the environment. Future research should test samples from upstream and downstream of the Lake Cumberland WWTP.

The other sampling site for which there was evidence that a WWTP is playing a role in increasing the prevalence of tet(B) was at West Hickman. There, the upstream sample indicated no presence of tet(B), while the effluent and downstream samples both indicated the presence of the gene. This evidence suggests that it is likely the WWTP that is introducing the gene to the environment here. Otherwise, it is likely that the gene would have been present upstream in addition to effluent and downstream of the WWTP.

There are several limitations to the research in this study. Some of the electrophoresis results were inconclusive. That is, the bands were very faint or not at the expected spot. Future research should try to clarify the findings. Additionally, the fact that ARGs could be naturally occurring adds a degree of uncertainty to the findings. Nothing was done in the experiment to ensure that any evidence of DNA was from anthropogenic influences. It is also unknown why the sample from Silver Creek that tested positive for blaSHV using the first set of PCR conditions and primer sets failed to test positive using the second set of PCR conditions and primers for blaSHV. Future research should explore any differences in the two sets of conditions and

primers and endeavor to answer why different results were obtained for PCR reactions of the same gene.

In conclusion, it is possible that some of the WWTPs involved in this study are increasing the prevalence of ARGs. Specifically, there is preliminary evidence that the WWTP at West Hickman is increasing the prevalence of tet(B). Also, there is some evidence that the WWTP at Lake Cumberland is increasing the prevalence of tet(B) and the WWTP at Silver Creek is increasing the prevalence of blaSHV. There was no evidence that any WWTP in the study are playing a role in increasing the prevalence of blaCTX. Future research should reproduce results, replicate ambiguous results, test for samples upstream and downstream of the WWTP at Lake Cumberland, and test for additional ARGs.

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Works Cited

1. **Chopra, I. & Roberts, M.** 2001. Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. Microbiol. MOl. Biol. Rev. 65(2):232. DOI: 10.1128/MMBR.65.2.232-260.2001.
2. **Asensi, M., Chagas, T.**, **Cury, J., Davila, A., Oliveira, J., Seki, L., & Silva, D.** 2011. Multiresistance, beta-lactamase-encoding genes and bacterial diversity in hospital wastewater in Rio de Janeiro, Brazil. Journal of Applied Microbiology. 111: 572-581. DOI:10.1111/j.1365-2672.2011.05072.x
3. **Kong, K.**, **Mathee, K., & Schneper,** L. 2009. Beta-lactam antibiotics: from antibiosis to resistance and bacteriology. APMIS 118: 1-36. DOI 10.1111/j.1600-0463.2009.02563.x
4. **Rollins, D. & Joseph, S.** 2000. Mechanism of Action of Beta-Lactam Antibiotics. URL: http://life.umd.edu/classroom/bsci424
5. **Bonnet, R.** 2004. Growing Group of Extended-Spectrum β-Lactamases: the CTX-M Enzymes. Antimicrobial Agents and Chemotherapy. 48(1): 1-14. DOI: 10.1128/AAC.48.1.1–14.2004
6. **Bonomo, R. & Paterson, D**. 2005. Extended-Spectrum β-Lactamases: a Clinical Update. Clinical Microbiology Reviews. 18(4): 657-686. doi:10.1128/CMR.18.4.657–686.2005.
7. **Bradford, P.** 2001. Extended-Spectrum β-Lactamases in the 21st Century: Characterization, Epidemiology, and Detection of This Important Resistance Threat. Clinical Microbiology Reviews. 14(4): 933-951. DOI: 10.1128/CMR.14.4.933–951.2001.

8. **Kehrenberg, C., S. A. Salmon, J. L. Watts, and S. Schwarz.** 2001. Tetracycline resistance genes in isolates of Pasteurella multocida, Mannheimia haemolytica, Mannheimia glucosida and Mannheimia varigena from bovine and swine respiratory disease: intergeneric spread of the tet(H) plasmid pMHT1. J. Antimicrob. Agents Chemother. 48:631–640.
9. **UCSF School of Medicine.** 2016. http://missinglink.ucsf.edu/lm/molecularmethods/pcr.htm
10. **Guess, B.** 2016 Gel Electrophoresis. Cellular Physiology. https://cellularphysiology.wikispaces.com/Gel+Electrophoresis
11. **Chuanwu, X., Foxman, B., Marrs, C., Nriagu, J., Simon, C., and Zhang, Y.** 2009. Prevalence of antibiotic Resistance in Drinking Water Treatment and Distribution Systems. Applied and Environmental Microbiology. 75: 5714-5718
12. **Bai, X., Li, J., Ma, X., Xiao, X., Xu, F., and Zhang, H**. 2015. The Drinking Water Treatment Process as a Potential Source of affecting the bacterial antibiotic resistance. Elsevier. 533: 24-31.
13. **Rosas, I., Martinez, L., Salinas, E., Cruz-Cordova, A., Espinosa, N., Gonzales-Pedrajo, B., and Amabile-Cuevas, CF.** 2015. Characterization of Escherichia coli Isolates from an Urban Lake Receiving Water from a Wastewater Treatment Plant in Mexico City: Fecal Pollution and Antibiotic Resistance. Current Microbiology. 71: 490-495.
14. **Cho, K., Kim, T., and Yi, T.** 2015. Fate and behavior of extended-spectrum beta-lactamase-producing genes in municipal sewage treatment plants. Journal of

Environmental Science and Health Part A-Toxic/Hazardous Substances & Environmental Engineering. 50: 1160-1168.

15. **Bergeron, S., Boopathy, R., Corbin, A., Lafleur, G., and Nathaniel, R.** 2015. Presence of antibiotic resistant bacteria and antibiotic resistance genes in raw source water and treated drinking water. International Biodeterioration & Biodegradation. 102: 370-374.
16. **Balcioglu, A., and Oncu, N.** 2013. Antimicrobial Contamination Removal from Environmentally Relevant Matrices: A Literature Review and a Comparison of Three Processes for Drinking Water Treatment. Ozone: Science & Engineering. 35: 73-85.
17. **Guo, M., Yang, J., and Yuan, Q**. 2015. Fate of Antibiotic Resistant Bacteria and Genes during Wastewater Chlorination: Implication for Antibiotic Resistance Control. PLoS ONE 10(3): e0119403.doi:10.1371/journal.pone.0119403
18. **Burgmann, H., Czekalski, N., and Diez, E.** 2014. Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake. The ISME Journal. 8: 1381-1390.
19. **Aali, R., Hassanzadeh, A., Khanahmad, H., and Nikaeen, M**. 2014. Monitoring and Comparison of Antibiotic Resistant Bacteria and Their Resistance Genes in Municipal and Hospital Wastewaters. International Journal of Preventive Medicine. 5: 887-894.
20. **Akiyama, T. and Savin, M**. 2010. Populations of Antibiotic-Resistant Coliform Bacteria Change Rapidly in a Wastewater Effluent Dominated Stream. Science of the Total Environment. 408: 6192-6201.
21. **Auerbach, E., McMahon, K., and Seyfried, E.** 2007. Tetracycline Resistance Genes in Activated Sludge Wastewater Treatment Plants. Water Research. 41: 1143-1151.

22. **Ashbolt, N., Jury, K., Khan, S., Stuetz, R., and Vancov, T.** 2011. Are Sewage Treatment Plants Promoting Antibiotic Resistance? Critical Reviews in Environmental Science and Technology. 41: 243-270.

23. **Lapara, T., Burch, T., Eichmiller, J., McNamara, P., Tan, D., and Yan, M.** 2011. Tertiary-Treated Municipal Wastewater is a Significant Point Source of Antibiotic Resistance Genes into Duluth-Superior Harbor. Environmental Science & Technology. 45: 9543-9549.

24. **Mao, D., Alvarez, Pl, Hou, J., Li, F., Luo, Y., Mu. Q., Rysz, M., Yang, F., and Yu, S.** 2015. Prevalence and Proliferation of Antibiotic Resistance Genes in Two Municipal Wastewater Treatment Plants. Water Research. 85: 458-466.

25. **Rahube, T., Koraimann, G., Viana, L., and Yost, C**. 2014. Characterization and Comparative Analysis of Antibiotic Resistance Plasmids Isolated from a Wastewater Treatment Plant. Frontiers in Microbiology. Vol. 5: 558.

26. **Moura, A., Correia, A., Henriques, I., Oliviera, C., and Smalla, K.** 2012. Broad Diversity of Conjugative Plasmids in Integron-Carrying Bacteria from Wastewater Environments. Federation of European Microbiological Societies. 330: 157-164.

27. **Johnson, B.** Occurrence of Antibiotic Resistant Genes in Proximity to Wastewater Treatment Plants. National Conference on Undergraduate Research. April 3-5, 2014. (Work conducted under Dr. Bill Staddon's supervision)

28. **Center for Disease Control (CDC).** 2014. Antibiotic Resistance Threats in the United States. US Department of Health and Human Services.

29. **National Institute of Health (NIH).** 2016. Estimates of Funding for Various Research, Condition, and Disease Categories. U.S. Department of Health and Human Services. URL: https://report.nih.gov/categorical_spending.aspx

30. **Barguigua, A.**, **Jamili, L., Otmani, F., Reguig, A., Talmi, M., and Timinouni, M.** 2016. Prevalence and genotypic analysis of plasmid-mediated β-lactamases among urinary *Klebsiella pneumoniae* isolates in Moroccan community. Journal of Antibiotics. Nature publishing group.