Enumeration of extended-spectrum-beta-lactamase-producing *Enterobacteriaceae* from downstream and upstream water samples close to a wastewater treatment plant in Hall County, Georgia, USA.

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Abstract

Extended-spectrum beta-lactamases (ESBLs) are a group of enzymes shown to rapidly evolve and confer resistance to a number of antibiotics, including beta lactams. Organisms that produce ESBLs pose both threats and challenges in the administration of appropriate agents to treat infections. ESBLs exhibit antibiotic resistance by destructing the antibiotics' structure and are typically found encoded on bacterial plasmids that can easily be transferred between bacteria from the family *Enterobacteriaceae*. Water environments such as streams can help the spread of antibiotic-resistant bacteria which can originate from a variety of sources, including wastewater treatment plants, agricultural sources, and residential septic tank systems. An ongoing study is currently looking into the isolation and identification of ESBL-producing *Enterobacteriaceae* from efferent and afferent water samples obtained from Flat Creek Water Reclamation, located in Hall County, Georgia. In this paper, we carried out enumeration of ESBL-producing bacteria from water samples obtained immediately upstream and downstream from the Flat Creek Water Reclamation facility and compare those samples with quality control performance standards. Surface water samples were obtained and ESBL-producing *Enterobacteriaceae* were isolated from both pre-treated water obtained within Flat Creek Water Reclamation and from downstream water samples, including ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae*. Coliforms were found both upstream and downstream from the water treatment facility.

Introduction

The spread of antibiotic-resistant bacteria (ARB) through the heightened use and misuse of antibiotics has become a growing problem in both clinical and environmental settings. Waterborne pathogens are just one of the ways in which antibiotic resistance is spread throughout communities, since certain aquatic environments such as creeks and streams can play a role as both a habitat and a transporter for this organisms (Kittinger et al., 2016), though the treatment of wastewater has a significant impact on the total number of coliforms present in the environment. Of particular interest are bacteria which produce extended-spectrum-beta-lactamases (ESBLs). ESBLs are enzymes which have the ability to hydrolyze the structure of beta-lactam antibiotics, breaking open the beta-lactam ring thereby inactivating the antibiotic (Medeiros, 1997). Beta-lactams are considered one of the most important methods for treating serious infections (Damoa-Siakwan, 2005). ESBLs are capable of conferring resistance to a number of antibiotics, including penicillin, aztreonam, and first-, second-, and third-generation cephalosporins. They are also inhibited by clavulanic acid. The number of known ESBLs now exceeds 200, and studies from over 30 different countries provide testament as to how widespread ESBL-producing organisms are (Paterson & Bonomo, 2005).

Primarily, ESBLs can be found produced by members of the family *Enterobacteriaceae.* Although *Enterobacteriaceae* are part of the natural intestinal flora of humans, they can also be pathogens capable of producing ESBLs (Zhang et al., 2015). Particular ESBL-producing *Enterobacteriaceae* include *Escherichia coli* and *Klebsiella pneumoniae*. The concern with aquatic environments is that bacteria from

different origins are able to mix and exchange antibiotic resistant genes (ARGs) which can then go on to contaminate municipal urban waters. As of now, there is no data published on the significance of ESBL-producing *Enterobacteriaceae* in the waters of North Georgia. Currently, researchers from the University of North Georgia, Gainesville, Georgia, are studying afferent and efferent water samples obtained from Flat Creek Water Reclamation, a water treatment facility located in Gainesville, Georgia, to analyze *Enterobacteriaceae* isolates for antibiotic susceptibility and confirm the presence of *bla* genes that encode for ESBLs. The objective of the present study was to enumerate surface water samples obtained from both inside and outside Flat Creek Water Reclamation in order to analyze the extent of ESBL prevalence in urban aquatic environments, including pre-treated and post-treated water.

Materials and Methods

Sampling Sites and Water Sample Collection

Between September and November of 2016, water sampling was conducted in Gainesville, Georgia. On 26 September 2016, afferent and efferent water samples were obtained from inside Flat Creek Water Reclamation. The remainder of the water samples, obtained between 4 and 16 November 2016, were collected upstream and downstream from the treatment facility, immediately outside its grounds along the creek. For each sampling, the water samples were collected at the water surface using sterile bottles (50 ml/bottle), then the collected water samples were immediately transported to our lab for further analyses within 1 h.

Microbiological Analysis

The afferent and efferent water samples obtained from Flat Creek Water Reclamation on 26 September 2016 were first diluted in a series of 9 ml saline tubes, then 100 µl from selected dilutions were plated on CHROMagar ESBL plates (CHROMagar, Paris, France) and incubated at 37℃ for 23 h.

The first of the water samples obtained from outside Flat Creek Water Reclamation were collected on 4 November 2016. Immediate upstream and downstream surface water samples were diluted in a series of 9 ml saline tubes, then 100 µl from all dilutions were plated on MacConkey and brain heart infusion (BHI) agar plates (Oxoid, Hampshire, England) and incubated at 37℃ for 24 h.

Another collection of both upstream and downstream water samples were obtained on 9 November 2016. Again, serial dilutions with 9 ml saline tubes were done, and 100 µl from all dilutions were plated on MacConkey and BHI agar plates. All plates were incubated at 37℃ for 114 h (with water bath placed in incubator to keep the plates from drying out).

Antimicrobial Susceptibility Testing and ESBL Confirmation

On 14 November 2016, water samples were collected upstream and downstream from Flat Creek Water Reclamation. On these samples, a presumptive most probable number (MPN) analysis was performed as follows: 10 ml of each sample was placed in a separate series of 5 double-strength lactose broth (DSLB) tubes; 1.0 ml of each sample was placed in a separate series of 5 single-strength lactose broth (SSLB) tubes; and 0.1 ml of each sample was placed in a separate series of 5 SSLB tubes. All 30 tubes (15 upstream, 15 downstream) were incubated at 35℃ for 24 h. After incubation, streak plates of 1 downstream and 1 upstream DSLB tubes were made on eosin methylene blue (EMB; Oxoid, Hampshire, England) agar plates to be incubated at 37℃ for 24 h.

Another sample collected on 16 November 2016 utilized the MPN method, this time including antibiotics in the lactose broth tubes. Ten µl of cefotaxime and cefepime were placed in each lactose broth tube

before pipetting the water sample into the tubes. The same series of 15 tubes were made for the upstream and downstream water samples and incubated at 35℃ for 22 h. On 17 November 2016, bacterial glycerol stocks were made by mixing 500 µl glycerol with 500 µl lactose broth from DSLB tubes (two upstream and two downstream) and placing the mixtures into 2 ml screw top cryogenic tubes.

On 5 December 2016, the four glycerol stocks were taken out of the freezer. HardyCHROM ESBL agar plates (Hardy Diagnostics, Santa Maria, CA) were streaked with a loopful from each glycerol stock. The remained of the thawed glycerol stocks were poured into DSLB tubes containing 10 µl cefotaxime each. The HardyCHROM ESBL plates and DSLB tubes were incubated at 37℃ for 72 hours. A new batch of bacterial glycerol stocks were made from the DSLB tubes and placed in the freezer for storage.

Results

Detection of ESBL-producing *Enterobacteriaceae*

The CHROMagar ESBL plates inoculated with water from within Flat Creek Water Reclamation showed growth on the ESBL plates with pre-treated water samples but no growth on the plates with post-treated water after 23 h of incubation. On the pre-treated water sample plate diluted to 1/10th the concentration of the original sample, a total number of 657 colony forming units (CFUs) were observed (Figure 1), of which 151 of those were identified as ESBL-producing *Klebsiella* (23%), 51 were ESBL-producing *E. coli* (8%), and 455 which were grouped together into a broad category of white to yellow colonies containing ESBL-producing *Acinetobacter* or ESBL-producing *Pseudomonas* (69%). Analytical interpretation of these results reveals that an average of 2.55 x 10^3 CFU/ml of ESBL-producing *E. coli* were present in the original sample of the pre-treated water within the facility, an average of 1.25 x 10^4 CFU/ml of ESBL-producing *Klebsiella* were present, and an average of 5.27 x 10^4 CFU/ml of other ESBL-producing microorganisms were found in the original sample of pre-treated water (Table 1).

Isolate	Avg. CFU/ml
Escherichia coli	2.55 x 10^3
***Klebsiella* spp.**	1.25 x 10^4
Everything else	5.27 x 10^4

Table 1. Average number of CFUs per ml of original pre-treated sample obtained from within Flat Creek Water Reclamation located in Gainesville, Georgia, USA.

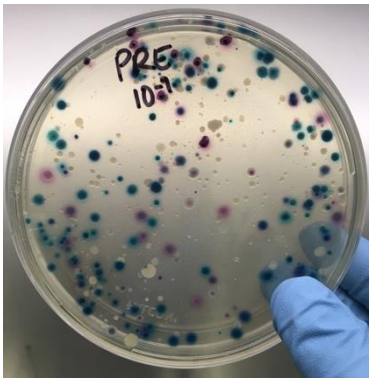

Figure 1. Colonies growing on CHROMagar ESBL plate inoculated with a diluted sample of pre-treated water.

BHI results from the sample obtained on 7 November 2016 showed an average of 5.67 x 10^2 CFU/ml in the original sample obtained from upstream of the treatment facility and 2.67 x 10^2 CFU/ml in the original downstream sample. Growth on the MacConkey agar plates correlated to an average of 3.33 x 10^1 CFU/ml upstream and 0.0 CFU/ml downstream. Results from BHI plates with upstream and downstream samples collected on 14 November 2016 showed an average of 1.9 x 10^3 CFU/ml upstream and an average of 3.33 x 10^2 CFU/ml downstream. Results from MacConkey plates inoculated with the same samples showed an average of 3.4 x 10^3 CFU/ml upstream and 3.33 x 10^1 CFU/ml downstream from Flat Creek Water Reclamation (Table 2).

Date	Sample Location	Avg CFU/ml for BHI agar plates	Avg CFU/ml for MacConkey agar plates
11/7/16	Upstream	5.67 x 10^2	3.33 x 10^1
	Downstream	2.67 x 10^2	0
11/14/16	Upstream	1.9 x 10^3	3.4 x 10^3
	Downstream	3.33 x 10^2	3.33 x 10^1

Table 2. Average number of CFUs per ml of surface water samples obtained upstream and downstream from Flat Creek Water Reclamation, located in Gainesville, Georgia, USA.

Antibiotic Susceptibility of ESBL-producing *Enterobacteriaceae*

Using the MPN method of determination, the first series of lactose broth tubes, inoculated with upstream and downstream water samples obtained on 15 November 2016, resulted as follows: gas production was observed in all 3 of the DSLB tubes, 2 out of 3 of the 1.0 ml SSLB tubes, and 0 out of 3 of the 0.1 ml SSLB tubes for the downstream water sample; and gas production was observed in all 3 of the DSLB tubes, 2 out of 3 of the 1.0 ml SSLB tubes, and 1 out of 3 of the 0.1 ml SSLB tubes for the upstream water sample. According to the Most Probable Number Procedure and Tables Guidebook published by the USDA (2014), the MPN of coliforms present in 100 ml of water was 93 for the downstream water sample and 150 for the upstream sample (Table 3). This series did not contain any antibiotics. One loopful of liquid from 1 downstream DSLB tube and 1 upstream DSLB tube were streaked on separate EMB agar plates and coliforms were confirmed to be present after 24 h incubation.

In the MPN test for the samples collected on 17 November 2016, 10 µl cefotaxime was placed in all of the lactose broth tubes for both the upstream and downstream MPN tests. There was only enough cefepime to place 10 µl in all of the downstream lactose broth tubes but only 2 out of 3 of the DSLB tubes for the upstream sample. After incubation, results for both the downstream and upstream water samples were the same, and gas production was observed in the following: all 3 of the DSLB tubes, all 3 of the 1.0 ml SSLB tubes, and 0 out of 3 of the 0.1 ml SSLB tubes. The MPN of coliforms present in 100 ml of water was 240 for both samples (Table 3). One loopful of liquid from 2 downstream and 2 upstream DSLB tubes each were plated on HardyCHROM ESBL agar plates and, after 72 h incubation, ESBL-producing *Klebsiella* was observed on 1 of the downstream plates and other ESBL-producing bacteria were observed on all 4 plates (Table 4).

Sample	NO. OF TUBES GIVING POSITIVE REACTION OUT OF 3 of 10ml per each	3 of 1.0ml per each	3 of 0.1ml per each	MPN index per 100ml	Confirmation of coliforms on EMB plates
Downstream	3	2	0	93	Yes
Upstream	3	2	1	150	Yes

Table 3. Results from the presumptive MPN test performed with samples obtained on 15 November 2016 upstream and downstream from Flat Water Creek Reclamation, located in Gainesville, Georgia, USA.

Sample	NO. OF TUBES GIVING POSITIVE REACTION OUT OF 3 of 10ml per each	3 of 1.0ml per each	3 of 0.1ml per each	MPN index per 100ml	Confirmation of ESBL-producers on ESBL plates
Downstream	3	3	0	240	Yes
Upstream	3	3	0	240	No

Table 4. Results from the presumptive MPN test containing cefotaxime and cefepime, performed with samples obtained on 17 November 2016 upstream and downstream from Flat Water Creek Reclamation, located in Gainesville, Georgia, USA.

Discussion

Positive results for coliforms and ESBL-producing *Enterobacteriaceae* were observed in all tests of this preliminary study. There were some limitations in this study: conflicts with obtaining samples within the grounds of Flat Creek Water Reclamation shortly after the beginning of the study led us to switch to sampling elsewhere on Flat Creek. Fortunately, we were able to obtain samples from upstream and downstream of the facility immediately outside the property. By the time this problem with sampling was resolved, temperatures going into the last months of the year had already fallen below ideal temperatures, however ESBL-producing *Enterobacteriaceae* could still be found in the last water samples. Isolates showed resistance to the third-generation cephalosporin cefotaxime and, importantly, showed resistance to cefepime, the fourth-generation cephalosporin, in both upstream and downstream water samples. Bacterial glycerol stocks were made for further study of these ESBL-producing microorganisms.

In summary, these findings indicate the possible contamination of ESBL-producing *Enterobacteriaceae* in North Georgia waters, though further analyses and broader research is needed. Water pollution may be closely related to not only the water treatment facility but also nearby animal farms and food processing plants which may be worth looking into for further study. Pinpointing the origins of certain ESBL-producing strains could prove beneficial in the treatment of bacterial infections and prevention of the dissemination of such bacteria. Sampling is planned for Spring 2017 to investigate further the prevalence of ESBL-producing *Enterobacteriaceae* in the waters of North Georgia.

References

Damoa-Siakwan, S. (2005). Extended-spectrum beta lactamases: an overview. *Journal of Infection Prevention, 6,* 25-28.

Kittinger, C., Lipp, M., Folli, B., Kirschner, A., et al. (2016). Enterobacteriaceae Isolated from the River Danube: Antibiotic Resistances, with a Focus on the Presence of ESBL and Carbapenemases. *PLoS ONE, 11,* 11.

Medeiros, A. (1997). Evolution and dissemination of β-lactamases accelerated by generations of β-lactam antibiotics. *Clinical Infectious Diseases, 24,* S19-S45.

Paterson, D. & Bonomo, R. (2005). Extended-Spectrum β-Lactamases: a Clinical Update. *Clinical Microbiology Reviews, 18(4),* 657-686.

USDA (2014). Most Probable Number Procedure and Tables. *USDA Food Safety and Inspection Service, Office of Public Health Science, Athens, GA.* PDF.

Zhang, H., Zhou, Y., Guo, S., & Chang, W. (2015). Multidrug resistance found in extended-spectrum-beta-lactamase-producing *Enterobacteriaceae* from rural water reservoirs in Guantao, China. *Frontiers in Microbiology, 6,* 267.