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Quantification of extended-spectrum-beta-lactamase-producing *Enterobacteriaceae* from water sources in Hall County, Georgia, USA

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Abstract: Extended-spectrum beta-lactamases (ESBLs) are a family of enzymes that confer resistance to a number of antibiotics, including those containing a beta lactam ring. ESBLs exhibit antibiotic resistance by destroying the antibiotic's structure and may be encoded by bacterial plasmids that can easily be transferred between bacteria from the family Enterobacteriaceae. Organisms that produce ESBLs pose both threats and challenges in the administration of appropriate therapeutic agents to treat infections. Water environments such as streams can help the spread of antibiotic-resistant bacteria which can originate from a variety of sources, including food processing, waste water treatment plants, and urban runoff. We are studying the isolation and identification of ESBL-producing *Enterobacteriaceae* from water samples obtained from a water treatment plant and its receiving stream in north Georgia. In this paper, we carried out enumeration of ESBL-producing bacteria from water samples obtained immediately upstream and downstream from the water treatment plant. ESBL-producing *Enterobacteriaceae* were observed from both pre-treated water obtained from the water treatment plant and from upstream (240/ 100 ml) and downstream (240/ 100 ml) water samples, including ESBL-producing *Escherichia coli* and *Klebsiella pneumonia*. Our results indicate that ESBL-producing *Enterobacteriaceae* are present in all water sources sampled. This suggests that the waste water treatment plant is not the source of these microorganisms. Further studies are needed to determine the originating source.

Antibiotics were one of the miracles of the 20th century and were used for treating several different common infections that were fatal without treatment. Repeated exposure to antibiotics can select for bacteria-containing genes that code for antibiotic inactivating enzymes. Although some bacteria naturally possess the genes for antibiotic resistance, other bacteria can acquire resistance via the uptake of DNA from the environment or by random DNA mutations (Lorenz and Wackernagel, 1994; Paterson and Bonomo, 2005). Therefore, common infections are now becoming harder to treat due to the frequency of occurrence of multi-drug resistant bacteria. According to the Center for Disease Control, more than two million people in the United States become infected with antibiotic resistant microbes each year (Centers for Disease Control and Prevention [CDC], 2017).

Enterobacteriaceae, such as *Escherichia coli*, are part of the natural intestinal microbiota of humans. They can also cause common infections such as GI tract distress. Most of these infections are easily treated with beta-lactam antibiotics (Damoa-Siakwan, 2005). However, some of these pathogens have gained antibiotic resistance due to acquisition of extended-spectrum beta-lactamases (ESBLs) (Zhang et al., 2015). ESBLs are enzymes that have the ability to hydrolyze the structure of beta-lactam antibiotics, breaking open the ring and thereby inactivating the antibiotic (Medeiros, 1997). ESBLs are primarily produced by

members of the family *Enterobacteriaceae*. They are capable of conferring resistance to a number of antibiotics, including penicillin, aztreonam, and first-, second-, and third-generation cephalosporins. ESBLs were first seen in the 1980s (Doi et al., 2016) and now exceed 200. Studies from Europe and Asia have documented the presence of ESBLs in wastewater, streams and wells (Canton et al., 2008; Brechet et al., 2014; Paterson & Bonomo, 2005).

The spread of antibiotic-resistant bacteria 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 these organisms (Kittinger et al., 2016). The concern with aquatic environments is that bacteria from different origins are able to mix and exchange antibiotic resistant genes. These modified bacteria can then go on to contaminate municipal urban waters, important sources for drinking and recreational activities (Doi et al., 2016). If ingested, these bacteria can potentially cause infection in humans as well as alter the normal gut microbiota (Blaak et al., 2015). Without drugs to treat infections caused by antibiotic-resistant microbes, results/infections could be fatal. Prior unpublished data from our lab has detected the presence of ESBL-producing *Enterobacteriaceae* in both local streams and waste-water treatment plant (McCuen et al, 2017). However, the impact of that finding is unknown without quantification.

The objective of the present study is to quantify ESBL-producing *Enterobacteriaceae* to determine the potential impact of waste water, the treatment plant, and environmental sources. We sampled water entering (pre-treated) and leaving (post-treated) a wastewater plant as well as up- and downstream of the release point from the plant. A study has shown that waste water treatment plants can significantly contribute to dissemination of ESBL-producing *Enterobacteriaceae* (Blaak et al., 2015). There is also data showing the presence of these bacteria in natural waters (Zhang et al., 2015). Therefore, we wanted to determine if a similar occurrence is found in the water bodies of north Georgia. Analysis of these samples could help identify potential contamination sources.

Materials and Methods

Sampling Sites and Water Sample Collection

We collected pre- and post-treated water on 26 September 2016 from a water treatment plant in north Georgia. We collected water approximately 300 meters upstream and downstream from the discharge from the plant on 4, 9, and 16 November 2016. For each collection date, the water samples were collected in triplicate at the water surface using sterile bottles (50 ml/bottle). The water samples were put on ice and transported to our lab for further analyses within 1 h.

Microbiological Analysis

The pre- and post-treated water samples from the water treatment plant were first diluted in a series of three 9 ml (10^{-1}, 10^{-2}, 10^{-3} dilutions) saline tubes, then 100 µl from all dilutions were plated on CHROMagar ESBL plates (CHROMagar, Paris, France) and incubated at 37°C for 23 hours.

The surface water samples immediately upstream and downstream from the treatment plant were diluted in a series of the 9 ml saline tubes (as above), then 100 µl from all dilutions were plated on MacConkey agar (Thermo Scientific™ Remel) and incubated at 37°C for 24 hours.

Most Probable Number (MPN) Analysis

Up- and downstream water samples were analyzed using plating and a presumptive most probable number (MPN) analysis with confidence intervals using standard methods as described (Oblinger and Koburger, 1975; USDA, 2014). MPN is a serial dilution method that is particularly useful for low concentrations of bacteria (Blodgett, 2010). Briefly, 10 ml of each sample was placed in a separate series of 3 double-strength lactose broth (DSLB) tubes; 1.0 ml of each sample was placed in a separate series of 3 single-strength lactose broth (SSLB) tubes; and 0.1 ml of each sample was placed in

Table 1: Average number of CFUs per ml of original pre-treated sample obtained from a water reclamation center located in north Georgia, USA.

Isolate		CFU/ml
Pre-treated		
	E. coli	1.51×10^4
	Klebsiella spp.	5.1×10^3
	Everything else	4.55×10^4
Post-treated		
	E. coli	undetected
	Klebsiella spp.	undetected
	Everything else	undetected

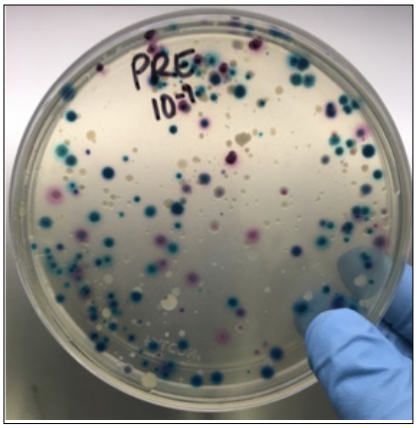

Figure 1. Colonies growing on HardyCHRO-Magar ESBL plate inoculated with a diluted sample of pre-treated water.

a separate series of 3 SSLB tubes. All 18 tubes (9 upstream, 9 downstream) contained a durham tube and the following antibiotics to select for the presence of antibiotic resistant gram negative bacteria: cefotaxime (50 µg/ml) and cefepime (50 µg/ml). These tubes were incubated at 37° C for 24 hours. The ratio of positive and negative tubes gives a confidence interval for the concentration of bacteria present per 100 ml of sample (Blodgett, 2010; Rice, 2012).

To confirm the presence of lactose fermenters, Eosin Methylene Blue (EMB) agar plates (Oxoid, Hampshire, England) were streaked using samples from gas positive tubes. ESBLs were confirmed using HardyCHROM ESBL agar plates incubated for 72 hours.

Results

Enumeration of ESBLs using spread plate technique

The HardyCHROM ESBL plates inoculated with water from within the treatment plant showed growth on the ESBL plates with pre-treated water samples (Figure 1) but no growth on the plates with post-treated water after 23 hours of incubation. On the pre-treated water sample plate diluted to 1/10 the concentration of the original sample, colony forming units (CFUs) were observed and calculated (Figure 1 & Table 1). Of these colonies, 23% were identified as ESBL-producing *Klebsiella*, 8% were ESBL-producing *E. coli*, and the rest were grouped together into a broad category of white to yellow colonies containing ESBL-producing Acinetobacter or ESBL-producing *Pseudomonas* (69%).

Enumeration of ESBL-producing Enterobacteriaceae *using MPN Analysis*

Stream samples collected on 4 and 9 November did not yield statistically significant number of colonies on HardyCHROM plates (Goldman and Green, 2008), but we confirmed the presence of *Enterobacteriaceae* using MacConkey

Table 2: Results from the presumptive MPN test containing cefotaxime and cefepime, performed with samples obtained on 16 November 2016 upstream and downstream from a water reclamation center, located in north Georgia, USA.

	Number of positive tubes in dilutions				
Sample	**10ml**	**1.0ml**	**0.1ml**	**MPN index per 100ml (Confidence interval)**	**Confirmation of ESBL-producers on ESBL plates**
Downstream	3	3	0	240 (36 – 1300)	Yes
Upstream	3	3	0	240 (36 – 1300)	Yes

agar plates. Therefore, we utilized MPN analysis (Oblinger and Koburger, 1975) specifically for 16 November water to inoculate a larger sample size. 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 contained gas bubbles. Based on the MPN chart, the antibiotic resistant coliforms present in 100 ml of water was 240/100 ml for both locations (Table 2; Oblinger, 1975). To confirm the presence of ESLBs, we inoculated one loop of culture from 2 downstream and 2 upstream DSLB tubes each of which were plated on HardyCHROM ESBL plates. After 72 hours incubation, ESBL-producing *Klebsiella* was observed on 1 of the downstream plates and other ESBL-producing bacteria were observed on all 4 plates (Table 2).

Discussion

In this study, we detected and quantified antibiotic resistant ESBL-producing *Enterobacteriaceae* up- and downstream of a water treatment plant. Our preliminary result indicate 240 ESBL-producing *Enterobacteriaceae* /100 ml of water sample. Thus, we have confirmed the presence of ESBL-producing *Enterobacteriaceae* in a north Georgia stream. Positive results for coliforms and ESBL-producing *Enterobacteriaceae* were observed in all tests of this preliminary study. 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. The MPN analysis showed there are potentially significant number of ESBL-producing *Enterobacteriaceae* present both upstream and downstream of the treatment plant. Additional studies involving water samples from different upstream and downstream locations will determine if this result is the norm. Collection dates were spread apart enough that temperatures during the November collection periods had already fallen below ideal temperatures; however ESBL-producing *Enterobacteriaceae* could still be found in the last water samples. Additional studies examining the number of ESBL-producing *Enterobacteriaceae* versus environmental temperature will be informative.

While the impact of these numbers is not yet known (Brechet et al., 2014), we have set a baseline for future water quality monitoring in this stream. A Dutch study of surface water and wastewater has found 220 CFU / 100 ml ESBL-producing *E. coli*. While their study specifically looked for one type of bacteria, we have enumerated all ESBL-producing bacteria present (Blaak et al., 2015). Interestingly, the numbers of ESBL-producing bacteria up- and downstream are similar. This suggests the water treatment plant does not contribute to stream contamination by these bacteria. Doi et al. suggests multiple sources that lead to introduction and spread of ESBL-producing *Enterobacteriaceae* in the environment including food animals, companion animals, wild birds and runoff in addition to waste water (2017). The presence of ESBL-producing *Enterobacteriaceae* may be related to nearby food processing plants and urban runoff, which may be worth looking into in the future. 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 2019 to investigate further the prevalence of ESBL-producing *Enterobacteriaceae* in the waters of north Georgia.

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Contributor Bios

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