

Extended-spectrum beta-lactamase-producing Enterobacteriaceae in Flat Creek Water Reclamation Center.

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Abstract

Extended-spectrum beta-lactamases (ESBLs) are enzymes that are capable of degrading frequently used beta-lactam antibiotics such as penicillin and cephalosporins. Organisms that produce these enzymes are often multi-drug resistant. ESBLs are commonly produced by the Enterobacteriaceae group of gram negative bacteria that are naturally occurring gut microbiota. Currently, ESBLs pose a significant health threat due to their ability to cause a multitude of difficult to treat infections, and are one of the leading causes of death world-wide. The *bla* genes that code for ESBL can be passed via horizontal transmission. As of yet, there is no data on prevalence of ESBL-producing Enterobacteriaceae in North Georgia water sources. Flat creek is located in North Georgia and runs directly into Lake Lanier, which is a main source of drinking water for the Atlanta area as well as a popular spot for water recreation. We obtained efferent and afferent water samples from Flat Creek Water Reclamation Center in Hall County Georgia. These water samples were filtered, grown in an enrichment media and plated on selective media that yielded the isolation of ESBL-producing Enterobacteriaceae. We found ESBL producing enterobacteria in all the samples we tested. Further identification methods revealed the presence of ESBL producing *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Citrobacter freundii* and *Escherichia coli*. Following identification, disk diffusion method was used to test susceptibility of the isolates to several commonly used antibiotics. This testing revealed that all isolates were multi-drug resistant. Lastly, DNA was extracted from the isolates

to confirm the presence of *bla* genes that encode for ESBLs by agarose gel electrophoresis.

Forty-two percent of the isolates were confirmed to be carrying one or more of the *bla* genes.

Introduction

Antibiotics are essential in modern medicine for fighting infectious diseases, and are vital to the survival of vulnerable patient populations with weakened immune systems. The overprescribing and misuse of antibiotics across human and animal populations has led to selection of antibiotic resistant bacteria (ARB). The consistent antibiotic pressure placed on bacteria facilitates ARB “superbugs” to thrive and flourish. (Chen et al., 2010). When common antibiotics become ineffective, increasingly toxic antibiotics are often the only option. Patients who acquire resistant infections face much higher morbidity and mortality rates. ESBLs are one such mechanism for bacteria to confer resistance. Extended-spectrum beta-lactamases (ESBLs) are enzymes that hydrolyze extended-spectrum cephalosporins with an oxyimino side chain, cleaving the beta-lactam ring, inactivating the antibiotic. The mechanism by which this occurs is rearrangement of the amino acid sequences of the active sites. (Patterson., 2003)

Since first identified in 1983, ESBLs have become increasingly prevalent because ESBL enzymes can be plasmid mediated, thus their resistant genes are easily transferred between bacterial strains via horizontal and vertical gene transmission. Resistances to β -lactams mediated by extended-spectrum β -lactamases (ESBL) are especially relevant among Enterobacteriaceae (Ojer-Usoz, 2014). Antibiotics are essential in modern medicine for fighting infectious diseases, and are vital to the survival of vulnerable patient populations with weakened immune systems. The overprescribing and misuse of antibiotics across human and animal populations has caused the selection of antibiotic resistant bacteria (ARB). This consistent antibiotic pressure placed on

bacteria facilitates ARB “superbugs” to thrive and flourish. (Chen et al., 2010). When common antibiotics become ineffective, increasingly toxic antibiotics are often the only option. Patients who acquire resistant infections face much higher morbidity and mortality rates.

Aquatic environments have a significant role in transmitting ARB to human populations.

Bacteria originating from numerous sources such as hospitals and livestock are washed into wastewater, via watershed and disposal methods, where they amalgamate and then the bacteria have the ability transfer resistance genes via horizontal transmission, through plasmid mediated genes. Consequently, water establishes an ideal path for the dissemination of resistant bacterial genes among human and animal populations, as well as naturally occurring bacterial ecosystems (Ojer-Usoz, 2014). Hospital waste compounds the problem of spreading resistance. Waste from patients colonized with ARB along with large amounts of antimicrobials are released into wastewater and represent a continuous selective pressure upon the ARB. Antimicrobial selective pressure favors horizontal transfer of resistance genes among and between different species of bacteria (Hocquet, 2016).

ESBLs are derivatives of the plasmid-mediated enzyme families of TEM (temoniera), SHV (sulfhydryl variable), and OXA (oxacillin) (Patterson , 2003). In recent years, CTX-M type enzymes have been increasing in prevalence and have superseded TEM and SHV derivatives and *Escherichia coli* has become the most prevalent species among ESBL-producing *Enterobacteriaceae* (Bethel, 2011). These genes can be transmitted through binary fission or by horizontal transmission via plasmids.

Materials and Methods

Sampling Sites and Water Sample Collection

Between August 2015 and March of 2016, water sampling was conducted at Flatwater Creek Water Reclamation Facility in Gainesville, Georgia. We collected 100 mL of both effluent and influent samples from the plant on August 2015, September 2015, and March 2016. 100 ml of water samples were collected from approximately 50 cm below the water surface using sterile bottles. The collected water samples were stored on ice for 30 minutes for transport to the lab.

Microbiological Analysis

For each sample, 100 mL of water was filtered through sterile 0.45 µm membrane filters (Nalage, Rochester, NY, USA). The filters were removed and placed in 20 mL of enterobacteria enrichment (EE) Broth (Oxoid LTD, Hampshire, England) and incubated in a shaking incubator at 275 rpm for 24 h at 37 °C. After incubation, a loop full of culture was streaked on an ESBL chromogenic Agar plates (Hardy Diagnostics, Santa Maria, CA) and incubated at 37°C for 24 hours. The chromogenic plates contain a proprietary blend of antibiotics to select for multi-drug resistant strains and allow for easy identification of E.coli and Klebsiella via the chromogens (pink and blue respectively). The colonies that grew on the chromogenic plates with different colors and morphologies were isolated, sub-cultured, and confirmed for multi-drug resistance. The antibiotic resistant enterobacterial species were identified by using API strips (Biomeriux) as per the manufacturer's instruction.

Antimicrobial Susceptibility Testing and ESBL Confirmation

We selected these specific antibiotics to be representative of increasing levels of resistance within the beta-lactam structured drugs as well as non-beta-lactam drugs. The higher the

generation, the more toxic the antibiotic. We used a first, second, third and fourth generation beta-lactams (Penicillin, Cefepime, Cefotaxime, Ceftazidime, Imipenem) as well as we used three beta-lactams with clavulanic acid (Augmentin, Ceftazidime/Clavulanic Acid, Cefotaxime/Clavulanic Acid) a known beta-lactamase inhibitor. We also used four non-beta-lactam antibiotics, (Chloramphenicol, Gentamycin, Tetracycline, Minocycline) that are common front line drugs.

The disk diffusion method was used to test susceptibility of the isolates against 12 anti-microbial agents. The tested antibiotic concentrations: Cefotaxime-Clavulanic Acid 30/10 μ g, Cefotaxime 30 μ g, Ceftazidime-Clavulanic Acid 30/10 μ g, Cefepime 30 μ g, Chloramphenicol 30 μ g, Imipenem 10 μ g, Minocycline 30 μ g, Tetracycline 30 μ g, and Gentamicin 120 μ g. Isolates showing resistance to three or more antibiotic classes were defined as multidrug resistant (MDR).

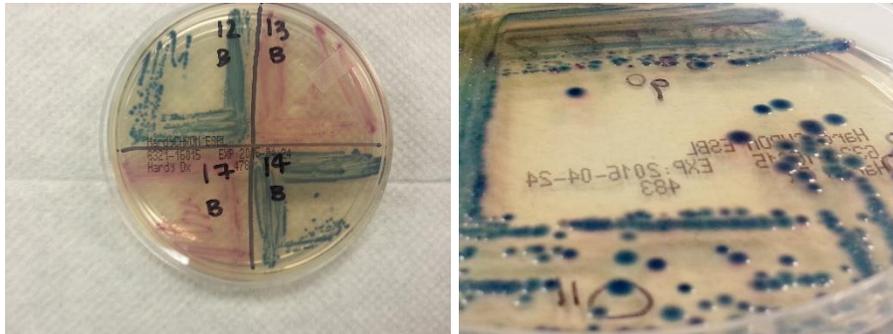
Polymerase Chain Reaction (PCR) to Detect *bla* Genes

We extracted genomic DNA from the bacterial isolates using a DNA extraction kit (Thermo Scientific). We performed PCR to detect *bla*TEM, *bla*CTX-M, *bla*SHV, and *bla*OXA genes using specific primers found in previously published work (Chen et al., 2010). We used a 1 μ g genomic DNA, 2.5 μ L of forward primer, 2.5 μ L of reverse primer, 19 μ L of sterile water, and one PCR bead (GE healthcare, UK), for a total reaction volume of 25 μ L

Results

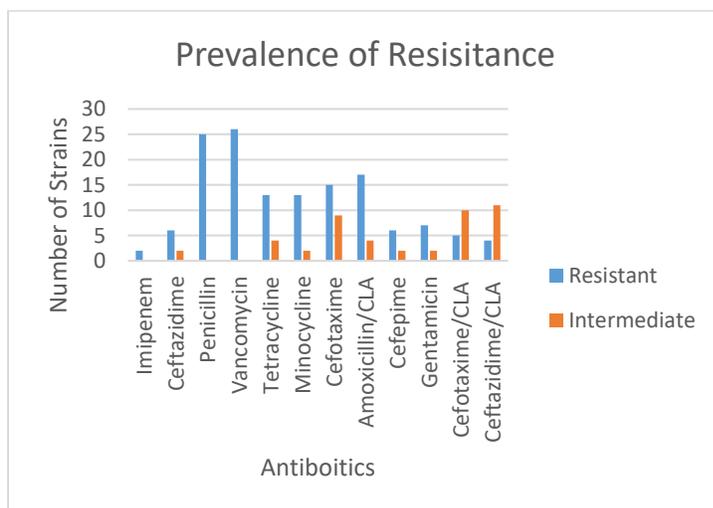
Variety of ESBL-Producing Enterobacteriaceae Identified. Multidrug resistant ESBL producing enterobacteria were found in both the efferent and afferent samples obtained in all three trials. 26 individual isolates were found to include *Ent. coloaecae* (3), *E. coli* (5), *Kl. Pneumoniae* (9), *Ent.*

intermedium (1), *Ent. Aerogenes* (4), *Ent. Agglom Ind+ Sac+* (1), *C. Freundii H2S-Ind* (1), *Ent. Agglom* (1). *Kluyvera Sp.*(1).



Antibiotic Resistance in Isolates

We found some resistance to every antibiotic tested. The resistance followed the outcome we would have expected, and 99.96% to penicillin. 57% of the isolates were resistant to the third generation cephalosporin cefotaxime, while only 23% were resistant to the fourth generation cephalosporin cefepime. 50% of the isolates were resistant to the second generation tetracycline minocycline. 23.08% were resistant to the non-beta-lactam antibiotic chloramphenicol. 7.69% were resistant to the carbapenem imipenem.



***bla* Genes Present in Isolates**

The *bla* gene is a plasmid encoding gene which codes for the production of an enzyme called beta-lactamase. Beta-lactamases come in four variations classified as types A, B, C, and D. The *bla* gene has been identified in four forms the TEM, SHV, CTX, and OXA genes. We found that 38.5% of all the isolates identified were positive for one of the four possible *bla* type gene. Of those, 50% of them were positive for two or more genes.

Bla Gene Type Number of isolates containing the gene Percentage of Isolates

SHV 4 15.4%

CTX 5 19.2%

OXA 3 11.5%

TEM 5 19.2%

Discussion

In this study, 26 individual isolates of ESBL-producing Enterobacteriaceae were found in both efferent and afferent water samples from the Flat Creek Water Reclamation Facility in Gainesville, Georgia. This facility treats water that comes from industrial, healthcare, and residential waste. The ESBL Enterobacteriaceae that we found were all multi-drug resistant. They showed increasing resistance from the penicillin based antibiotics up through the third and fourth generation cephalosporins, as well as, resistance to the carbapenem. We also had

resistance to the non-beta-lactam antibiotics showing that these isolates have developed more than one mechanism of resistance as beta-lactamase would have no effect on a drug without a beta-lactam ring in its structure. This suggests that these strains have been subjected to multiple selective pressures being in the constant presence of antibiotics, thus causing them to become resistant to higher order drugs.

There were some limitations in this study: There were only three trials performed over the course of a year which may not be representative of the entire picture. One of the trials was done during February, which turned out to be a very cold day, and that is the reason we believe we got far fewer isolates out of that trial.

We have given but a snapshot of the entire issue concerning ESBL in our community. In future studies we plan to look at the correlation between anthropological impact and the species and enumeration of ESBL producing bacteria. We also plan to look at possible seasonality to explain the reasoning for the winter data being significantly less than spring/summer.

ESBL Research References

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