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Incidence of antibiotic resistance and plasmid content in freshwater beach sand and water and clinical urinary tract infection Escherichia coli isolates

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Incidence of antibiotic resistance and plasmid content in freshwater beach sand and water
and clinical urinary tract infection *Escherichia coli* isolates

An Abstract of a Thesis in Biology

By

Robert White

Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Arts

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Antibiotic-resistant (AR) bacteria have been found in environmental ecosystems including beach sand and water, and pose a serious threat to the mitigation of human and animal disease. The presence of antibiotic residues in the environment, fueled by wastewater effluent and agricultural runoff, may produce selective pressure on introduced microbes such as *Escherichia coli,* leading to the production of AR populations. This study characterized and compared the antibiotic resistance patterns and plasmid content of *E. coli* isolated from a freshwater beach and clinical urinary tract infection (UTI) samples. A higher level of antibiotic resistance was expected in clinical (UTI) *Escherichia coli* isolates due to stronger selective pressure. A total of 171 beach isolates (comprised of 66 sampled from nearshore water and 105 sampled from sand) and 111 UTI isolates were screened by disk diffusion for microbial resistance to 11 antibiotics. More clinical UTI *E. coli* isolates were resistant to one or more of the antibiotics used in this study than were *E. coli* isolated from freshwater beach sand and adjacent near shore water ($p < 2.2^{-16}$). Although previous studies have shown higher instances of antibiotic resistance in beach sand isolates than those from adjacent water, none of the *E. coli* isolated from sand showed resistance to any of the antibiotics tested. Eight percent of water isolates and 49% of UTI isolates demonstrated resistance to at least one antibiotic. Significant correlation was found between resistance to one or more of the antibiotics used in the study and plasmid content in UTI isolates, but there was no correlation between antibiotic resistance and plasmid content in isolates from sand or water. The data suggests that the *Escherichia coli* isolated from sand, lacking the selective pressure of antibiotic residues typical in clinical and wastewater environments, could represent members of naturalized populations.

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Water and Clinical Urinary Tract Infection *Escherichia coli* Isolates

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1.0 Introduction:

Bennett Beach is a public beach in Erie County, New York frequented by recreational bathers. It was closed for 6 days in 2016 and 14 days in 2017 due to unsafe levels of *E. coli* in the water (Jackson 2019), demonstrating the impact of *E. coli* contamination on public health. Previously a significant number of *E. coli* were detected in dry sand 6 meters above the swash zone, and also in beach water (unpublished data). Beach sand and water *E. coli* isolates were again sampled in 2016 and 2017 for a subsequent study in which genetic sequence analysis of 16S ribosomal RNA (rRNA) could not determine intraspecific differences between sand and water populations (Jackson 2019). The characterization of antibiotic resistance and plasmid content of these sand and water populations at Bennett Beach, and comparison to the levels of antibiotic resistance in *E. coli* isolated from local clinical urinary tract infection samples may contribute to better understanding of the existing Bennett Beach microbial populations.

Escherichia coli (*E. coli*) is a facultative anaerobe which inhabits the hypoxic environment found in the lower intestines of humans and animals in addition to non-host ecosystems such as recreational water (Jang et al. 2017). *E. coli* is the microorganism most commonly associated with urinary tract infections (UTIs), having been the most frequently isolated species of 1,641 samples screened (66.4%) in one study (Reis et al. 2016).

Freshwater lakes, rivers, and streams harbor *E. coli* in both their water columns and soil sediments (Mauro et al. 2013; Ishii et al. 2006). The pathogenic capacity of *E. coli* poses a threat to public health: bathing in recreational waterways

can serve as a conduit for the transmission of pathogenic bacterial strains to humans (Leonard 2015). Gastrointestinal illness, for example, is the malady most frequently contracted from recreational bathing in the presence of added bacterial pathogens (O'Flaherty et al. 2019).

Sources of introduced *E. coli* vary. Municipal wastewater treatment plant (WWTP) effluent discharge (Servais and Passerat 2009), animal wastes, and agricultural run-off (Alm et al. 2003) are often culprits for the deposit of *E. coli* in beach sand and water, and seagulls and other shore birds in particular are known to deposit *E. coli* in sand (Whitman and Nevers 2003). Strains of bacteria from both human and non-anthropogenic sources have been found in the water column of streams and larger waterways (Perchec-Merien and Lewis 2012) and wet sand at freshwater beaches can also act as a reservoir for *E. coli* (Alm et al. 2003). Recurrent populations of *E. coli* found in temperate climate soil, sediment, and sand suggest that these strains have become naturalized or indigenous to the microbiota of these habitats (Ishii and Sadowsky 2008). The ability of bacteria to become naturalized in a beach sand environment may be due to the availability of nutrients, UV light protection, temperature moderation, and moisture retention (Beversdorf et al. 2006).

The proliferation of antibiotic-resistant bacteria pose a serious threat to the mitigation of human and animal disease. Rates of resistance to Ciprofloxacin as high as 54.9% have been found in uropathogens (Choe et al. 2018). Antibiotic-resistant bacteria also have been found in water, food, and environmental ecosystems (Wellington 2013). The presence of added antibiotic-resistant bacteria may promote the spread of microbial resistance to existing microbial populations, threatening

public health (Martinez 2008). Obayiuwana et al. (2018) found approximately 86% of bacteria isolated from pharmaceutical wastewater were resistant to multiple antibiotics, and similar results were found for bacteria in wastewater discharged from municipal wastewater treatment plants (WWTPs), indicating that community sewage promotes the release of antibiotic-resistant organisms into the environment (Botts et al. 2017; Zhang et al. 2009). Blaak et al. (2015) found that 27.6% of *E. coli* in rivers in the Netherlands were resistant to at least one antibiotic, as were 42% of *E. coli* in the Seine River (Servais and Passerat 2009). The capability of *E. coli* to spread to non-host ecosystems through the shedding of feces has established it as the leading fecal indicator bacterium for the detection of drinking and recreational water contamination, a role which it continues to fulfill. The ability of added *E. coli* to survive in non-host environments and spread disease demonstrates the importance of effective monitoring and study of *E. coli* in recreational waterways to ensure public health and safety.

The addition of antibiotic-resistant *E. coli* populations to natural ecosystems can increase the spread of resistance traits to pathogenic and non-pathogenic bacteria (Kraemer et al. 2019). Antibiotic-resistant organisms may also comprise part of the microbial community harbored in beach sand (Alm et al. 2006), especially where release of antibiotics in WWTP effluent and municipal sewage occurs (Marti et al. 2013; Samaraweera et al. 2019). Therefore, it is important to understand the antibiotic resistance profiles of microbial communities found in recreational waterways. It will become a serious threat to public health if the level of antibiotic resistance in

environmental *E. coli* populations reaches the same degree of resistance found in clinical *E. coli.*

Antibiotics can be excreted unchanged after human and animal consumption (Zhang et al. 2009), and their presence in the environment is also fueled by overuse in livestock for growth promotion and infection treatment (Pruden et al. 2006). Selective pressure can be expected to amplify antibacterial resistance in the environment as it does in clinical settings. If antibiotics enter the environment as chemical pollutants in human and agricultural wastes they can provide selective pressure for antibiotic resistance to introduced *E. coli* populations. And as the concentration of antibiotics in marine and freshwater waterways grows the steady selective pressure that antibiotic residues exert on resident bacteria may help produce naturalized populations of antibiotic-resistant organisms. Alm et al. (2014) found that of 147 *E. coli* isolated from nearshore water and foreshore sand at freshwater recreational beaches 19% (28) showed resistance to one or more of 16 antibiotics, and further suggested that long standing populations of antibiotic resistant *E. coli* may have become naturalized to these reservoirs. Bacterial mechanisms common to the proliferation of antibiotic resistance and naturalization to non-host environments warrant further study to improve the ability to monitor pathogenic and resistant populations that threaten public health. One such mechanism frequently found in *E. coli* is plasmid content.

Plasmids, extra-chromosomal DNA molecules capable of rapid translocation between organisms (Botts et al. 2017), play an important role in the evolution of bacterial genomes (Sherratt 1982). *Enterobacteriaceae* containing plasmids have been established as critical drivers of antibiotic resistance spread in clinical settings where

antibiotic usage is high by facilitating horizontal transfer of resistance genes (San Milan 2018). Plasmids acquire genes through mobile elements such as insertion sequences and transposons, and their ability to transmit these genes to numerous hosts makes them excellent vectors for the spread of antibiotic resistance (Rozwandowicz et al. 2018). There are three primary methods of horizontal transfer: conjugation, transduction, and transformation (Lerminiaux and Cameron 2019). The horizontal transfer of antibiotic resistance genes on conjugative plasmids magnifies the spread of drug resistant phenotypes throughout bacterial colonies (Lekunberri et al. 2017). Plasmid-mediated antibiotic resistance occurs with high frequency in the human gut (San Milan 2018) but plasmid exchange has also been found in beach sand microcosms (Alm et al. 2014).

An analysis of wastewater treatment plant effluent found 21 different plasmid-mediated resistance gene cassettes in bacterial isolates, including those encoding chloramphenicol-resistance proteins and β-lactamases (Tennstedt et al. 2003), indicating the presence of plasmids in natural environments. Plasmid mediated β-lactamase production contributes to the proliferation of antibiotic-resistant *E. coli* (Ramos et al. 2013), and plasmid-mediated Ciprofloxacin resistance has been shown to occur in conjunction with β-lactamse resistance (Domokos et al. 2019).

When antibiotic resistance genes are plasmid-mediated horizontal transfer may cross phylogenetic barriers increasing their spread, and their threat to public health (Al-Shamarti and Mohsin 2019). Fluoroquinolones are commonly used to treat urinary tract infections (UTIs), and *qnrA3* genes encoding fluoroquinolone resistance have been found on plasmids in both clinical and environmental bacterial species even in the absence of exposure to these antibiotics (Michon et al. 2011). Genes that

encode for resistance to two other UTI antibiotics, Sulfamethoxazole (*sul)* and Trimethoprim *(dfr),* have been found in wastewater effluent, suggesting these antibiotic-resistance genes predominate on plasmids (Suhartono et al. 2016). Plasmid-mediated transfer of Kanamycin resistance was identified in sand microcosm *E. coli* (Alm et al. 2014)*,* indicating the potential for spread of antibiotic-resistance traits in natural environments. The release and transfer of antibiotic resistance genes mediated by transmissible plasmids into natural environments poses a serious health risk (Botts et al. 2017) by further spreading antibiotic resistance.

The purpose of this study was to characterize and compare the antibiotic resistance patterns of *E. coli* isolated from beach sand, adjacent near shore-water, and clinical UTI isolates obtained from a local hospital, and to determine if there was a positive correlation between their antibiotic resistance and plasmid content. Relatively high resistance to Fluoroquinolones (39.0 - 54.9%) and Cephaolosporins (42.5 – 49.4%) have been found previously in clinical UTI isolates (Choe et al. 2018). This study found relatively lower levels of resistance in clinical UTI samples to the Cephalosporin Cephalothin (19%), and the Fluoroquinolone Ciprofloxacin (17%). The presence of antibiotic residues in the environment and the resulting selective pressure for resistance has led to the reporting of multi-drug resistance in Great Lakes beach sand as high as 5.4% (Alm et al. 2014). Similar levels of multi-drug resistance were anticipated in this study. All of the isolates in this study were screened for resistance to 11 antibiotics. The antibiotics were chosen based upon their affinity for 5 common target sites: Cell Wall synthesis (Ampicillin, Cefotaxime, Cefuroxime, Cephalothin, Imipenem); DNA synthesis (Ciprofloxacin); Cell

Membrane function (Polymixin); Protein Synthesis (Chloramphenicol, Nitrofurantoin, Tetracycline); and other metabolites such as Folic Acid (Sulfamethoxazole/Trimethoprim). Ampicillin and the Cephalosporins are from the β-lactam drug class, which are among the antibiotics most frequently prescribed in clinical applications. Resistance to fluoroquinolones such as Ciprofloxacin merits particular review, as they are often prescribed as medications of last resort for a variety of ailments including respiratory and urinary tract infections. It is important to monitor the dynamics of antibiotic-resistant *E. coli* populations in clinical and environmental settings given the danger to public health and safety that the spread of antibiotic resistance poses.

2.0 Methods

2.1 Bacterial Cultures:

Environmental *Escherichia coli* isolates (n = 171) that had been collected from a previous study of their virulence factor content (Jackson 2019) at Bennett Beach in Erie County NY (42°39'37.6"N 79°03'44.0"W) during the Summer of 2016 and 2017 were used in this study (Table 1 and Figure 1). These samples were drawn from two nearshore water and two dry sand sites 6m up from the swash zone, regions frequented by recreational bathers, and stored at -80°C in nutrient broth containing 20% Glycerol. These sampling sites are also located within close proximity to Erie County Sewer District No. 2 Big Sister Creek Wastewater Treatment Plant (WWTP) which discharges into Big Sister Creek and subsequently empties into Lake Erie at Bennett Beach

Clinical *E. coli* isolates (n = 111) from known urinary tract infection (UTI) samples were obtained previously from a hospital in Buffalo, NY (Pettibone, personal communication) and stored as above. All isolates were resuscitated by inoculating frozen cells into EC MUG Broth (Difco, BD Biosciences) followed by incubation in a circulating water bath (Thermo Scientific) for 22 hours at 44.5°C. Those isolates that grew at 44.5°C, produced gas, and demonstrated a blue-white fluorescence under long wavelength (366nm) UV light were considered putative *E. coli.* These isolates were streaked for isolation on MacConkey Agar (Difco, BD Biosicences) and incubated for 22 hours at 35°C to further confirm their identity as *E. coli.* Pink colonies that produced a haze of precipitated bile on MacConkey Agar were used for antibiotic

testing and plasmid DNA extraction (see below). Because the identity of the hospital or the patients from which UTI samples were obtained was not known, it was not considered necessary to obtain any permissions to use the UTI samples for this study.

2.2 Antibiotic Resistance Testing:

Antibiotic susceptibility was determined using the Kirby-Bauer disk diffusion method (Hudzicki 2009). After growth on MacConkey agar all *E. coli* strains were streaked for isolation on T-Soy agar (Difco, BD Biosciences) and incubated overnight at 35° C. Following incubation 4-5 well isolated colonies were picked using a sterile wire loop and inoculated into 8 mL of sterile 0.85% saline to achieve an optical density equal to that of a 0.5 McFarland standard (~1.5 x 10^8 CFU/mL) which was determined using a Biolog model 21907 turbidity meter (Biolog, Inc.) Within 15 minutes of preparation the solution was applied with a sterile cotton swab to the surface of a plate containing 25 mL Mueller-Hinton agar (Difco, BD Biosciences). Each plate was swabbed three times with the same swab, rotating the plate approximately 60 degrees after each swab application to achieve confluent growth of the bacteria. Eleven commercially available disks, each containing a different antibiotic, were applied to the surface of two Mueller-Hinton plates (five disks on one plate and six disks on a second plate) using a self-tamping disk dispenser (BD Sensi-Disk Dispenser, BD260661). The antibiotics and the working antibiotic concentrations to be used in the study are shown in Table 2. Within 15 minutes after application of the disks the plates were incubated at 37°C for 20 hours after which the diameter of the zones of growth inhibition were measured to the nearest millimeter

using a Measy 2000 150 mm caliper (Bel-Art Products). Zone diameters were

interpreted as Susceptible, Intermediate, or Resistant according to the European

Committee on Antimicrobial Susceptibility Testing Reading Guide (2012).

Intermediate results represent antibiotic concentrations associated with an unspecified

therapeutic effect (Rodloff et al. 2008) and in this study Intermediate results were

classified as Susceptible. Various parameters have been utilized for the definition of

multiple drug resistance (MDR) in previous studies. Resistance to at least one anti-

microbial agent in three or more antimicrobial categories was chosen as the MDR

criteria for this study (Magiorakos et al. 2012). *Escherichia coli* ATCC 25922 is a

recommended reference strain for antibiotic susceptibility testing, and both it and *E.*

coli K12 (ATCC#25404) served as controls.

2.3 Plasmid DNA Extraction:

All beach water (n = 66) and sand (n = 105) isolates and a subset of 64 UTI

isolates were screened for plasmid content. *E. coli* isolates were inoculated into

flasks containing 10 mL of LB broth and grown on an Innova benchtop orbital shaker

(Eppendorf North America Inc.) for 14 hours at 35°C with shaking (~206 rpm).

Monarch Plasmid DNA Miniprep Kit (New England Biolabs) was utilized for

plasmid DNA extraction following the manufacturer's protocol. Extracted plasmids

were separated by gel electrophoresis using 1.0% TAE agarose and run at ~94V for

~45 minutes. Gels were stained for 30 minutes in a 1 µg/mL ethidium bromide

solution followed by a 30 minute rinse in deionized water. Stained gels were

visualized using a Gel Doc EZ Gel Imager (Bio-Rad) and Image Lab software (Bio-

Rad).

2.4 Statistical Analysis:

The correlation between antibiotic resistance and isolate origin (sand, water, and UTI) was determined using a Chi-squared test. A T-test was used to compare the difference between the mean number of antibiotic-resistant UTI and freshwater beach samples. The mean number of water and sand plasmids was compared using a T-test. Chi-squared was used to test for correlation between antibiotic resistance and plasmid content in water isolates. All statistical analysis was conducted in R (R Core Team 2014).

3.0 Results

3.1 Sand and Water Isolate Antibiotic Resistance:

Escherichia coli isolates (n = 171) from freshwater beach water (n = 66) and sand (n = 105) were tested for resistance to 11 antibiotics (Figure 2A). Resistance to at least one antibiotic was detected in five of sixty-six water isolates (8%). None of the 105 sand isolates demonstrated antibiotic resistance. Most prevalent in *E. coli* water isolates was resistance to Cephalothin, which was detected in four out of five isolates (80%), followed by resistance to Ampicillin in three out of five isolates (60%), Tetracycline resistance in two of five (40%), and Chloramphenicol in one (20%).

Three distinct drug resistance profiles emerged in this study (Table 3). Only one water isolate resistant to antibiotics met the criteria for multiple drug resistance (MDR); it demonstrated resistance to Tetracycline, Cephalothin, and

Chloramphenicol. Three isolates were resistant to both Ampicillin and Cephalothin, and one isolate was resistant to a single drug, Tetracycline. Four of the five antibiotic-resistant water isolates (80%) were collected from Site 3 (Water/North), and one was collected from Site 2 (Water/South).

3.2 UTI Isolate Antibiotic Resistance:

Disk diffusion indicated that 54 (49%) of the 111 UTI isolates were resistant to at least one antibiotic. Unlike water *E. coli*, none of the UTI isolates demonstrated resistance to Chloramphenicol. In *E. coli* UTI isolates, resistance to Ampicillin was most prevalent (41%), followed by Cephalothin (19%), Tetracycline (18%), Ciprofloxacin (17%), Sulfamethoxazole/Trimethoprim (10%), Cefuroxime (4%), and Nitrofurantoin (1%) (Figure 2B).

Twenty-one (19%) of antibiotic-resistant UTI *E. coli* met the criteria for MDR. Twelve (11%) UTI isolates were resistant to three of the antibiotics tested, six (5%) were resistant to four antibiotics, and three (3%) were resistant to five antibiotics (Figure 2C).

3.3 β-Lactam and Quinolone Resistance:

The β-lactam drug class includes Ampicillin and the Cephalosporins, which are among the antibiotics most frequently prescribed in clinical applications. Fluoroquinolones such as Ciprofloxacin are often prescribed as medications of last resort for a variety of ailments including respiratory and urinary tract infections, and these two drug classes warrant particular attention. Water and UTI *E. coli* exhibited resistance to the β-lactam antibiotics Ampicillin, Imipenem, and three Cephalosporins

(Cephalothin, Cefuroxime, and Cefotaxime) included in this study. The most prevalent drug-resistance profile found in water isolates was *E. coli* resistant to both Cephalothin and Ampicillin (Table 3). Four UTI isolates resistant to Cefuroxime were also resistant to Ampicillin. None of the *E. coli* from freshwater beach water or UTIs were resistant to Imipenem or Nitrofurantoin.

There were no *E. coli* resistant to the fluoroquinolone Ciprofloxacin in freshwater beach water isolates whereas nineteen UTI isolates were resistant to Ciprofloxacin.

3.4 Comparison of Antibiotic Resistance in Water, Sand and UTI *E. coli:*

E. coli isolated from the clinical environment were more likely to be resistant to the antibiotics used in this test that environmental isolates: a chi-square test of independence showed that there was a strong correlation between location (sand, water, and UTI) and antibiotic resistance ($\chi^2 = 86.5$, $df = 2$, p value $< 2.2^{-16}$). None of the *E. coli* isolated from sand were resistant to any of the 11 antibiotics in this test. Significantly more clinical UTI *E. coli* isolates were resistant to one or more of the antibiotics used in this study than were *E. coli* isolated from freshwater beach sand and adjacent near shore water ($t = 9.4$, $df = 176$, $p < 2.2^{-16}$).

3.5 Plasmid Content:

Overall, 57 of 171 (33%) freshwater beach sand and water isolates contained plasmids (Figure 3A). Sand isolates were more likely to contain plasmids than those obtained from water ($p = 0.02$). Forty percent of sand isolates (42 of 105) and twenty-three percent of water isolates (15 of 66) contained plasmids. Two of the five

antibiotic resistant water isolates (40%) contained plasmids. Of 54 UTI isolates that demonstrated resistance to one or more antibiotics, 44 contained plasmids (81%).

Comparison of Plasmid Content in Water, Sand and UTI *E. coli.*

UTI *E. coli* were more likely to contain plasmids than *E. coli* isolated from water or sand ($t = 14$, $df = 234$, $p < 2.2^{-16}$). There was a strong correlation between antibiotic-resistant UTI isolates and plasmid content, and no correlation between antibiotic resistance and plasmid content in sand or water isolates ($\chi^2 = 33$, $df = 2$, $p = 6.8^{-8}$) [Table 3].

4.0 Discussion:

The escalation of antibiotic resistance can be attributed in part to over-prescription (Walker, 2012) and clinical misuse (Alothman et al. 2016). Higher incidences of antibiotic resistance in bacteria isolated from clinical settings is generally expected due to their higher antibiotic density (Struelens 1998). In this study 49% of UTI isolates exhibited resistance to at least one of the eleven antibiotics tested. These findings are consistent with those of Karami et al. (2017) wherein 40% of *E. coli* isolated from UTIs were resistant to any one of thirteen antibiotics. Resistance to Ampicillin was most frequent: 41% of UTI *E. coli* were resistant to Ampicillin, which was higher than the 27% Ampicillin resistance found in clinical isolates by Alm (2014). Cephalothin resistance was the second most prevalent, as 19% of total UTI isolates tested were resistant. Tetracycline (18%) resistance was third (Figure 2D).

This study found no antibiotic resistance in 105 *E. coli* isolated from dry sand (Figure 3). This result differs from a previous study of freshwater beach sand utilizing *E. coli* drawn from wave washed foreshore sand in which 5.4% of isolates demonstrated multiple drug resistance (Alm et al. 2014). Moist wave washed sand may be more conducive to bacterial growth, and this could account for the presence of antibiotic-resistant microorganisms in Alm's 2014 study. The dry sand sampled at Bennett Beach is less hospitable to *E. coli* due to reduced moisture retention, but this site was chosen because it is where beachgoers spend much of their time.

Five of the sixty-six isolates sampled from two water sites (8%) were resistant to at least one of the antibiotics tested. Four of these five were obtained from the North site which is closest to the Lake Erie estuary of Big Sister Creek, a warm water, low gradient stream bordered by agricultural land and low density residential development (New York State Department of State, Office of Planning and Development 1987). Storm water and sewage effluent treated at Erie County Sewer District No. 2 Big Sister Creek Wastewater Treatment Plant (WWTP) are discharged into Big Sister Creek, which empties into Lake Erie at Bennett Beach (Figure 1). Wastewater pollution is a significant contributor of bacteria to marine and freshwater environments (Zhang et al. 2015); therefore, wastewater treatment plants couple anthropogenic sources of antibacterial-resistant *E. coli* to the freshwater environment (Marti et al. 2013). The outfall from Big Sister Creek WWTP has been described as located only "a few thousand feet" from Lake Erie (Erie County Government 2017) and is a possible source for the antibiotic-resistant isolates found at the North site.

Resistance to Cephalothin, a first generation Cephalosporin commonly used since the 1960s, was most prevalent in water isolates. Eighty percent of antibiotic-resistant water *E. coli* isolates demonstrated resistance to Cephalothin (Figure 2A). Farrar and Krause (1970) attribute Cephalothin resistance to inducible β-lactamase production in two *Enterobacter* strains, but concede that permeability barriers in these cells may also be factors. Reis et al. (2016) also comment on the inherent ability of *E. coli* resistance due to this barrier. A future study using Farrar and Krause's methods for qualitative detection of β-lactamase activity could be conducted to determine this.

Water isolates presented three distinct drug resistance profiles, the first demonstrating resistance to a single drug, Tetracycline. Resistance to a broad spectrum antibiotic such as this is not surprising, as Tetracycline has been used commercially since the 1970s. The second profile demonstrated resistance to both Ampicillin and Cephalothin, and was the predominant resistance profile in water isolates, present in three of the five resistant *E. coli* isolates. The third profile, which demonstrated resistance to Tetracycline, Cephalothin, and Chloramphenicol, included the only isolate that met the criteria for multiple drug resistance in water *E. coli*. Chloramphenicol has been banned for veterinary use in food animals since the 1980s (Bischoff et al. 2004), which suggests that the source of Chloramphenicol resistance may not be agricultural runoff. In this study 1% of beach isolates were resistant to Tetracycline, and 2% were resistant to Ampicillin (Figure 2C). Alm et al. found 6% resistance to Tetracycline and 26.5% resistance to Ampicillin in beach isolates in a 2014 study which classified Intermediate results as resistant.

Escherichia coli isolated from sand possessed a significantly greater occurrence of plasmid DNA content (40%) than *E. coli* isolated from water (23%) even though these sand isolates did not demonstrate resistance to any of the antibiotics used in this study. Genes that express essential traits are seldom found on plasmids (Tazzyman and Bonhoeffer 2015), but there is evidence of the presence on plasmids of genes coding for functions beneficial to the host under specific environmental conditions, such as darkness (Nagarajan et al. 2013), or the presence of chemical elements, such as heavy metals (Kothari et al. 2019). The high levels of plasmid content found in these sand isolates may be explained by the need for the horizontal exchange of metabolic components which facilitate the beach sand population's transition to this particular environment. Genes coding for reductases necessary for anaerobic respiration in marine *Dinoroseobacter shibae* have been identified on plasmids (Ebert et al. 2013) suggesting that *Escherichia coli* could also demonstrate a broad spectrum of metabolic capabilities enabling survival in sand and water environments. Genetic sequence analysis of the plasmid content of these isolates was not performed in this study but in the future could determine if such genes are present. Plasmid extraction methods utilized in this study were limited to plasmids 25kb or less in size. Previous research has concluded that large *E. coli* plasmids can carry a variety of genes (Williams et al. 2013). It would be worthwhile to re-examine our isolates for the presence of larger plasmids in a future study.

The fluoroquinolone Ciprofloxacin, which interferes with DNA gyrase and isomerase function in gram-negative bacteria such as *E. coli* (Fournier et al. 2000), is a frontline antibiotic used in hospitals against urinary tract infections (UTIs), one of

the most frequently diagnosed healthcare infections (Richtel 2019).   A study by
Hitzenbichler et al. (2018) found that 10.5% of *E. coli* isolates obtained from
discharged patients with histories of prior hospital admissions demonstrated
resistance to Ciprofloxacin, lower than in this study, which found a resistance rate of
17% to Ciprofloxacin in UTI *E. coli.* A higher than expected rate of resistance to
Ciprofloxacin, especially in *E. coli*, represents a recent public health concern: Reis et
al. (2016) found a rate of Ciprofloxacin resistance in UTI *E. coli* more than double
that of other bacterial species (22.4% vs. 8.9%). Alm et al. (2014) found 5% of
clinical *E. coli* were resistant to Ciprofloxacin, and no resistance to Ciprofloxacin in
freshwater beach isolates. This study also found no resistance to Ciprofloxacin in
sand or water isolates in contrast to the relatively high level of resistance to this drug
found in UTI isolates. These higher incidences of Ciprofloxacin-resistant *E. coli*
isolated from clinical settings suggest that selective pressure there has influenced
resistance in these populations. The absence of resistance to this drug in beach sand
may mean added *E. coli* populations may become naturalized to the beach sand
environment which lacks this level of selective pressure.

Mathematical modeling found that pathogenic bacteria that are resistant to
multiple antibiotics pose greater risks to public health by limiting treatment options
(Tepekule et al. 2017). Clinical *E. coli* demonstrated seventeen different drug-
resistance UTI profiles in this study, with resistance ranging from one to five
antibiotics. Twenty-one MDR UTI isolates (19%) were found, a greater percentage
than the MDR which occurred in this study (<1%), and 5.4% of *E. coli* sampled from
a freshwater beach by Alm et al. (2014). The difference in MDR between clinical

and beach *E. coli* isolates supports the suggestion that selective pressure imposed by antibiotic residues impacts drug resistance. Resistance to antibiotics in the clinical environment is an adaptation that raises the fitness level of individual *E. coli* and when antibiotic resistance genes are encoded on plasmids their potential horizontal mobility can benefit entire microbial populations (Sobecky et al. 1997).

A strong correlation was found between plasmid content and antibiotic resistance in 54 UTI isolates resistant to any of the antibiotics used in this study ($p =$ 0.0003). Overall, 81% of *E. coli* isolated from UTIs contained plasmid DNA. All of the MDR isolates in this study contained plasmids, a finding consistent with that of Johnson et al. (2012) in which MDR was most commonly associated with plasmid content in a 2,202 human and avian *E. coli* isolates. Antibiotic-resistant UTI *E. coli* were significantly more likely to contain plasmids than antibiotic-resistant water *E. coli*. There was no correlation between plasmid content and antibiotic resistance in water *E. coli* isolates. Antibiotic resistance genes (ARG), can be prevalent in the presence of strong antibiotic use (Dimitriu et al. 2019) and lacking when such use is absent (Subbiah et al. 2011), which suggests an explanation for this discrepancy. This result further supports the importance of selective pressure on antibiotic resistance, and that the lack thereof in environments such as beach sand may contribute to the naturalization of added populations of *E. coli*.

The high level of antibiotic resistance in UTI isolates in this study is both expected and indicative of the effect of strong selective pressure on drug resistance. The proximity of the Big Sister Creek Wastewater Treatment Plant (WWTP) to the sampling site of the water isolates in this study suggests that a lesser degree of

selective pressure resulted in the antibiotic resistance found in these isolates.

Monitoring fecal indicator bacteria to gauge public safety is based on the presumption that added fecal bacteria result from contamination. Evidence exists that *E. coli* populations grow and persist in freshwater beach sand in the Great Lakes region (Alm et al. 2006; Ran et al. 2013; Whitman and Nevers 2003). Alm et al. (2014) found that *E. coli* populations in freshwater beach sand can demonstrate antibiotic resistance. This study found no antibiotic resistance in a similar *E. coli* population. The lack of antibiotic resistance in isolates sampled from dry sand where deposit from nearshore water is less likely and antibiotic residues are not as prevalent as in water and clinical sample sites suggests that these sand *E. coli* populations could be naturalized. The plasmids they carry may code for survival tools better suited to the dry sand environment than plasmids which would express antibiotic resistance traits. This comparison of antibiotic resistance patterns of environmental and clinical isolates suggests that the *Escherichia coli* isolated from sand may have adapted to an environment lacking the selective pressure of antibiotic residues typical in clinical and wastewater environments.

5.0 Figures and Tables

Figure 1. Four sampling sites at Bennett Beach, Angola on the Lake, NY, from which *Escherichia coli* isolates were obtained. (Not to scale, note the proximity of Site 3 to the outlet of Big Sister Creek.) Modified from Jackson (2019).
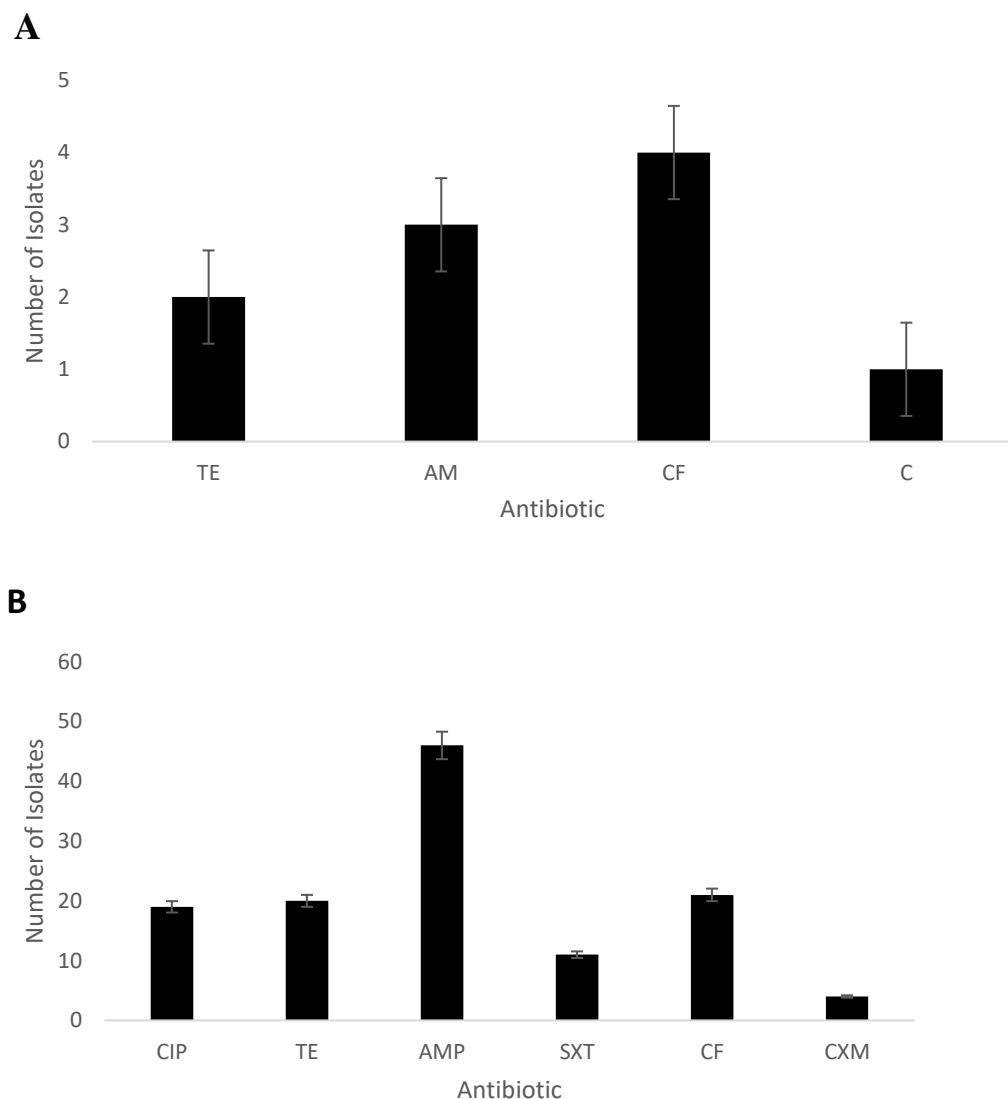
A

B

Figure 2. (A) Actual Number of *E. coli* freshwater beach water isolates resistant to each of 4 of the antibiotics used in the study. The highest frequency of resistance was to the β-lactam types Ampicillin and the first generation Cephalosporin Cephalothin (CF). All 3 of the *E. coli* isolates which demonstrated resistance to Ampicillin were also resistant to Cephalothin. (B) Actual number of clinical UTI *E. coli* isolates resistant to 6 of the 11 antibiotics used in this study: Ciprofloxacin (CIP); Tetracycline (TE); Ampicillin (AMP); Sulfamethoxazole/Trimethoprim (SXT); Cephalothin (CF); Cefuroxime (CXM); and Chloramphenicol (C) [A – B]. The highest frequency of resistance was to the β-lactam types Ampicillin and the first generation Cephalosporin Cephalothin. Each of the UTI *E. coli* isolates resistant to Ciprofloxacin also demonstrated resistance to at least 1 other antibiotic.

A

B

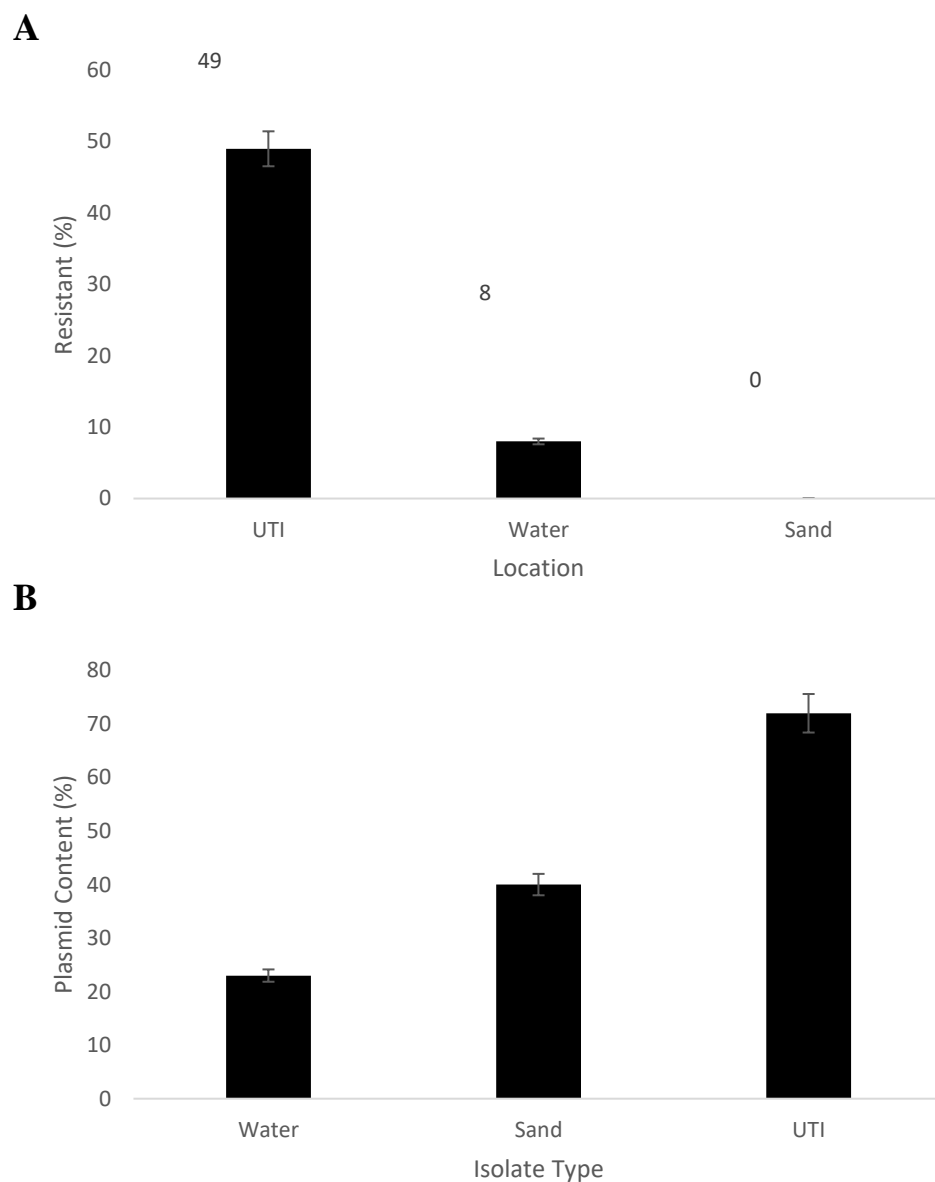**Figure 3.** (A) Percent of *E. coli* UTI, water, and sand isolates resistant to the antibiotics used in the study. (B) Percent of *E. coli* UTI, water, and sand isolates containing plasmid DNA. There was no correlation between plasmid content and antibiotic resistance in water isolates, and strong correlation between plasmid content and antibiotic resistance in UTI isolates ($\chi^2 = 33$, $df = 2$, $p = 6.8^{-8}$).

Table 1. Breakdown of the four sites by type (water/sand), location (Water/Sand), and number of *Escherichia coli* isolates recovered from Bennett Beach, Angola, NY.

Site	Type/Location	No. of Isolates
2	Water/South	30
3	Water/North	36
9	Sand/North	60
13	Sand/South	45
Total		171

Table 2. The 11 antibiotics in concentrations compliant with Clinical Laboratory Standards Institute (CLSI) standards used in the determination of Susceptibility/ Resistance of *Escherichia coli* environmental and clinical isolates.

Antibiotic	Concentration	Type	Target
Ampicillin	10 µg	Aminopenicillin	Cell Wall Synthesis
Cefotaxime	30 µg	Cephalosporin	Cell Wall Synthesis
Cefuroxime	30 µg	Cephalosporin	Cell Wall Synthesis
Cephalothin	30 µg	Cephalosporin	Cell Wall Synthesis
Chloramphenicol	30 µg	Chloramphenicol	Protein Synthesis
Ciprofloxacin	5.0 µg	Fluoroquinolone	DNA Synthesis
Imipenem	10 µg	Carbapenem	Cell Wall Synthesis
Nitrofurantoin	100 µg	Nitrofuran	30S Ribosomal Subunit
Polymixin B	300 IU	Polymixin	Outer Membrane
SMX/TMP*	23.75µg/1.25µg	Sulfamide	Folic Acid Synthesis
Tetracycline	30 µg	Tetracycline	30S Ribosomal Subunit

*Sulfamethoxazol/Trimethoprim

Table 3. Incidence of antibiotic resistance (R) in environmental water isolates to Tetracycline (TE), Ampicillin (AM), Cephalothin (CF), and Chloramphenicol (C). The Total column on the far right indicates the single or (multiple) drug resistance (DR) total of each isolate. The Total row at the bottom indicates the number of isolates resistant to each individual antibiotic.

Site	Antibiotic				DR Total
	TE	AM	CF	C	
Water (2)	R				1
Water (3)		R	R		2
Water (3)		R	R		2
Water (3)		R	R		2
Water (3)	R		R	R	3
Total	2	3	4	1	

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