"The Blue Devil Resistome": Antibiotic Resistance Transfer from the Environment to the Lab at Duke University

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Abstract

It is well observed that bacteria are becoming increasingly resistant to existing antibiotics worldwide. Horizontal gene transfer (HGT), more specifically, conjugation is one of the mechanisms by which bacteria gain antibiotic resistance. From 2017 to 2018, a project run by the You lab at Duke University collected bacterial samples from multiple areas on Duke's East and West campuses in an effort to map out a microbiome of the university. Environmental isolates that tested positive for antibiotic resistance were found, but studies have not been done to determine if these isolates could pose potential health threats to humans. By exposing the bacterial isolates to a series of antibiotic tests and measuring their growth, this project found that the conjugation of antibiotic resistance genes from environmental isolates to lab strains of *Escherichia coli* can occur. In addition, certain locations, like a door handle to a lab hallway door, expressed higher incidences of HGT capable bacteria than other locations. The ability of these isolates to transfer their resistance genes to lab strains would indicate a potential danger to the Duke population, as existing non-resistant pathogens could take on resistance to antibiotic treatments. In the future, understanding the mechanisms behind what drives these observations allows for the creation of better methods of combating antibiotic resistance and its spread.

Introduction

Modern antibiotics have been used to prevent life-threatening infections since the use of penicillin started in the 1940s (Ventola, 2015). Since then, other classes of antibiotics have been developed and used to prevent and minimize the severity of microbial infections, thereby extending lifespans (Rossolini, 2014). However, with each newly introduced antibiotic on the market, resistant bacteria have been discovered within only a couple decades of antibiotic use (CDC, 2013). Antibiotic resistant bacteria (ARB) have been on the rise globally and cause major health concerns and economic costs (Spellberg and Gilbert, 2014; Ventola, 2015). Methicillin-resistant Staphylococcus aureus (MRSA) kills more Americans every year than HIV/AIDS, Parkinson's disease, emphysema, and homicide combined (Golkar et al., 2014). Furthermore, multi-drug resistant pathogens are becoming increasingly prevalent (Golkar et al., 2014). In particular, carbapenem-resistant and extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae, a family of gram-negative bacteria that are resistant to beta lactam antibiotics, are becoming major threats as they are resistant to many or most antibiotic treatments (CDC, 2013). Many of the infections caused by ARB are acquired through health care facilities due to the frequent use of antibiotics and the high concentration of sick and vulnerable people. This results in nearly 99,000 deaths in the United States per year (Klevens et al., 2007). The total economic cost of treating ARB infections can be as high as $20 billion in health care costs (Golkar et al., 2014).

The incline of ARB has mainly been attributed to overuse, agricultural practices, lack of new antibiotics and regulatory barriers (Ventola, 2015). Antibiotic overuse, commonly due to over-prescription, leads to selecting for ARB by killing susceptible bacteria and leaving the resistant bacteria to survive and proliferate (zur Wiesch et al., 2011). This drives the evolution of resistance in bacterial species (zur Wiesch et al. 2011). Outside of the clinic, the agricultural

industry commonly uses antibiotics as a preventative measure and as a growth treatment for livestock to keep them healthy and give farmers larger yields (Michael et al., 2014). As a result, the antibiotics select for ARB within the livestock which are then ingested by humans in undercooked meat, increasing the risk of contracting ARB infections (CDC, 2013). A more likely means of ARB exposure due to agricultural use is through the environment as up to 90% of the antibiotics given to livestock are excreted and spread in the groundwater (Bartlett et al., 2013). As clinicians and the food industry continue to contribute to the increase in antibiotic resistance, this issue is exacerbated by the decline of the discovery of new antibiotics. There are barriers to doing research in antibiotic discovery due to increased regulation of clinical trials and high costs (Bartlett et al., 2013; Piddock, 2012).

Furthermore, resistance genes can be transferred from bacterium to bacterium via horizontal gene transfer, further exacerbating this issue (Read and Woods, 2014). This can occur in three different ways: transformation through the uptake of DNA from the environment, transduction by transferring genes using viruses, or conjugation where genes are transferred directly between bacteria via plasmids or transposons (Read and Woods, 2014). Although ARB in the environment have existed long before the use of antibiotics for human health and many of these resistant microbes are not harmful to humans, the potential for resistant genes to transfer to pathogenic species of bacteria is of concern.

In the 2017-2018 school year, a team of undergraduate students and graduate student mentors at Duke University collected microbial samples of key locations on Duke University's campus. The goal of the project was to discover the frequency of ARB throughout the university and determine hotspots that are likely to host ARB, with the hypothesis that resistance genes would more likely be found near the medical center, the student health center, and labs that work with ARB. This project was inspired by an analysis of microbial DNA diversity in New York City

subway stations done by Afshinnekoo et al. in 2015. The study found an abundance of ARB throughout the stations, although many of the bacteria were deemed non-pathogenic to humans. In a similar vein, many antibiotic resistant isolates were found throughout Duke University's campus, but it was unclear if these isolates could pose threats to human health. This project evaluated the ability of collected ARB isolates to transfer their resistance genes to a lab strain of *Escherichia coli*, a common human pathogen, through conjugation. Demonstration of this ability would indicate concern for human health as naturally harmless ARB could transfer resistance to pathogens, rendering those infections as difficult to treat. The knowledge gained from this project could allow for future implementation of potential interventions that could slow the spread of drug resistance based on the analysis of geospatial patterns of ARB observed on Duke University's campus.

Methods

Site Selection

The swabbing sites were selected to provide a basis of the variety of different areas that could harbor antibiotic resistant bacteria on campus. The sites were selected based on one of two factors: high traffic and exposure to antibiotics. Figure 1 depicts the locations of the selected sites.

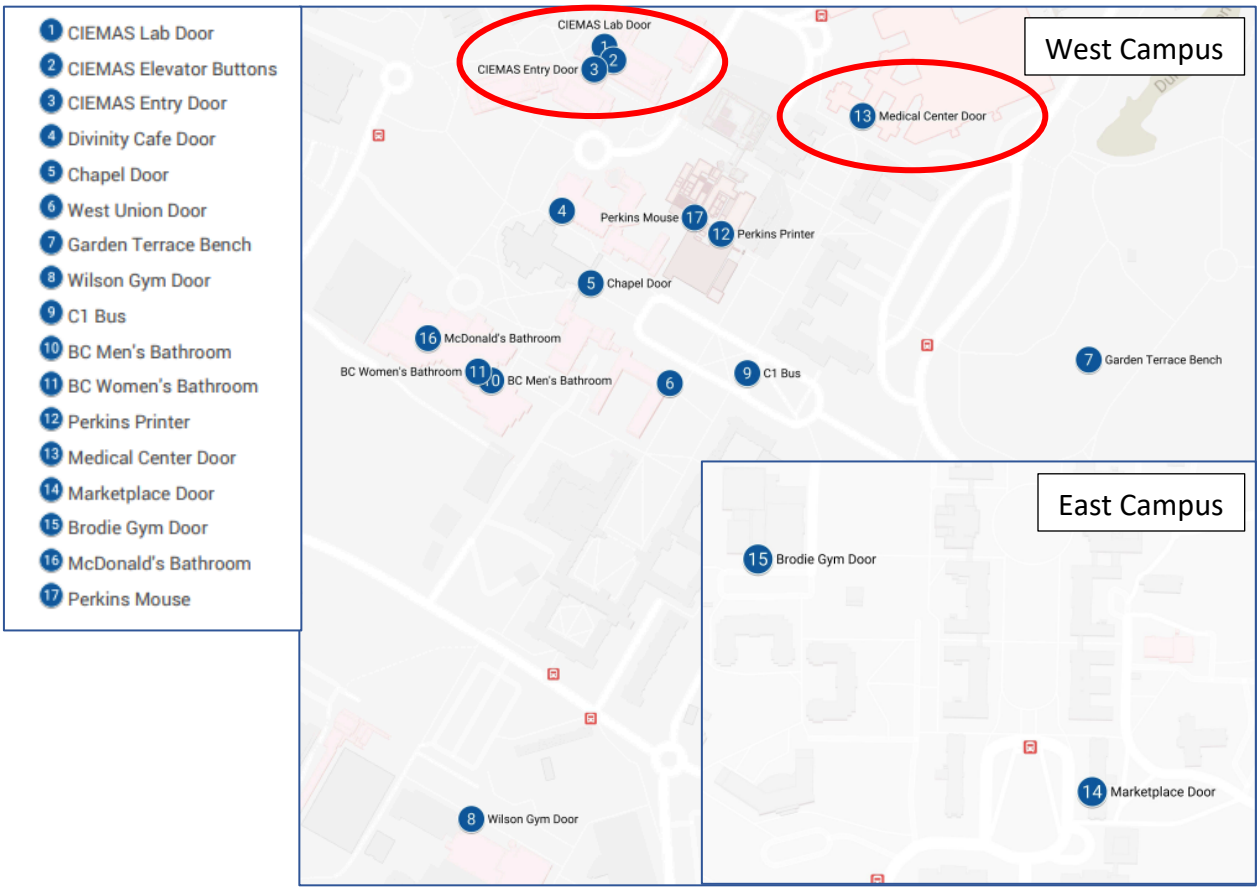

Figure 1: Locations of Sampling Sites. Each number represents a site of interest on Duke's campus that was swabbed for bacteria. The sites were generally chosen to obtain a representative sample of the microbiome at Duke in relation to human contact. The sites circled in red are sites chosen to represent areas close to the Duke Medical Center, which are expected to have a higher number of antibiotic resistant isolates collected.

Swabbing

Over the course of 11 months between June 2017 and May 2018, each of the sites were sampled once a week, totaling to 37 times. Each week, each site was swabbed twice for one minute each using an Eswab kit (Copan Inc). The surfaces chosen for swabbing were common areas that come into contact with people, such as door handles or buttons. The first swab was used to pick up

bacteria to be used for culturing and isolating antibiotic resistant bacteria that would then undergo whole genome sequencing. The same surface was swabbed again to represent the full bacterial load of the location which then underwent 16S rRNA sequencing for identification. Short-term storage of samples (plates with growth) were done at -20°C, while long term storage of samples was done at -80°C.

Culturing

Immediately after returning from sampling runs, the first swab sample from each site was plated in 6 different conditions: Tryptic Soy (TS) at room temperature (RT) and at 37°C, TS with cefotaxime (CTX) at RT and at 37°C, MacConkey at 37°C, and MacConkey with CTX at 37°C. TS agar is a general medium that allows growth of non-fastidious bacteria that do not have specific nutritional requirements, giving us the total microbial load. MacConkey agar was used to select for gram-negative enteric bacteria to provide us with the data for bacteria that can be found inside the human body. 4 μg/mL concentration of the broad-spectrum antibiotic CTX was used. After the swab samples were plated, the plates were allowed to grow for up to 96 hours in their respective temperature conditions and colony counts were performed every 24 hours.

Isolation

After 96 hours, unique colonies on the plates were isolated (based off of size, color, shape, and other distinct morphologies) and were allowed to grow for an additional 96 hours. After isolation, a homogenous individual colony was chosen to be cultured in TS broth then stored at -80°C.

Strain Characterization

Isolates were grown at 30°C for 24 hours and tested for resistance to 70 μg/mL of chloramphenicol (Cm), 100 μg/mL of carbenicillin (Carb), and horizontal gene transfer (HGT) ability (Figure 2).

Escherichia coli strain DA102 was used as the recipient for the HGT test. The HGT test relied on two assumptions:

1. DA102 is susceptible to Carb but contains chromosomal resistance to Cm (note that chromosomal resistance cannot be transferred through HGT)
2. The environmental isolate analyzed is susceptible to Cm but resistant to Carb.

If both of these conditions were met and there was growth in the Carb + Cm condition, it indicated that HGT had occurred. Growth was measured using optical density (OD) values.

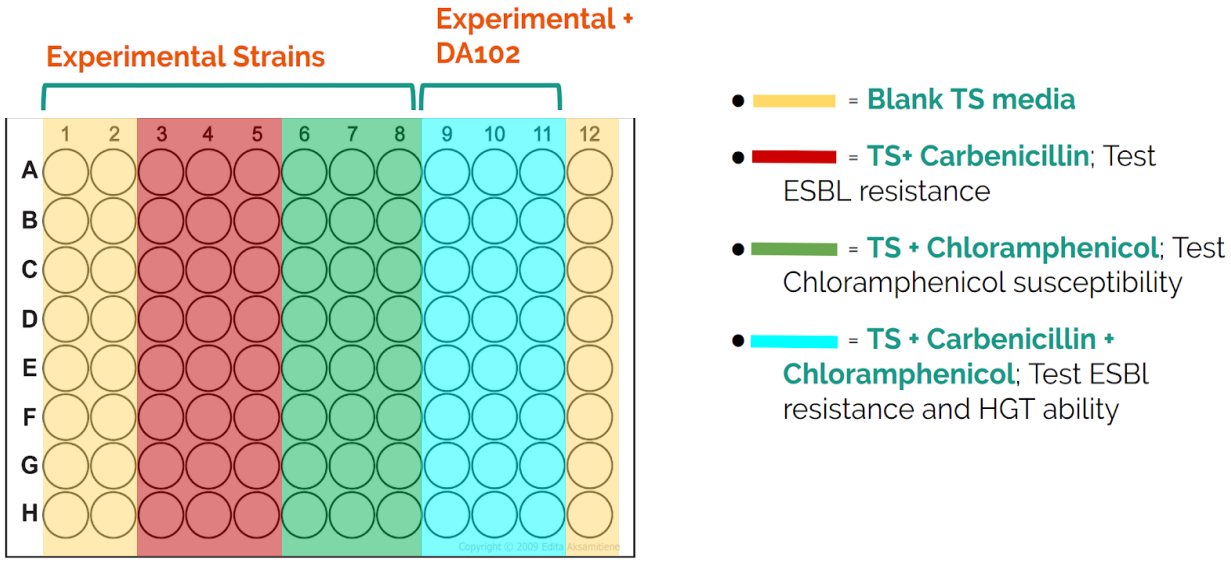

Figure 2: Strain Characterization Setup. Each row contained a different experimental strain collected from the environment while the columns were replicates of different selection conditions. Columns 1-2 measured growth in Tryptic Soy (TS) broth, columns 3-5 tested growth in TS broth with 100 µg/mL of carbenicillin, columns 6-8 tested growth in TS broth with 70 µg/mL of chloramphenicol, columns 9-11 tested growth of DA102 transconjugants (a strain of Escherichia coli that has chromosomal resistance to chloramphenicol), and column 12 was a negative control. I focused on the presence of growth in columns 9-11 for experimental strains that presented

resistance to carbenicillin and susceptibility to chloramphenicol, which indicated horizontal gene transfer ability.

Statistical analysis

Growth rate analysis from the strain characterization data was done in MATLAB. The data was split by strain and growth conditions and the values were averaged. The values of conditions that expected growth were then subtracted with the blank values from the control (no growth). These averaged and blank subtracted curves of the Optical Density vs Time were plotted. This was then utilized to calculate the growth rates of the strains in the different conditions by calculating the natural log of the curve. The maximum slope over time was found and reported as the growth rate. Isolates were considered to be resistant if their growth rate surpassed 0.1.

Results

I characterized several phenotypic aspects of 139 isolated bacterial strains. From these, there was evidence of strains on Duke University's campus which are resistant to Carb, a beta-lactam antibiotic. There was also evidence of strains that are resistant to an entirely different class of antibiotics, specifically Cm. Of these 139 strains characterized, 54 exhibited ESBL traits, 9 exhibited Cm resistance, and 30 exhibited HGT ability (Figure 3).

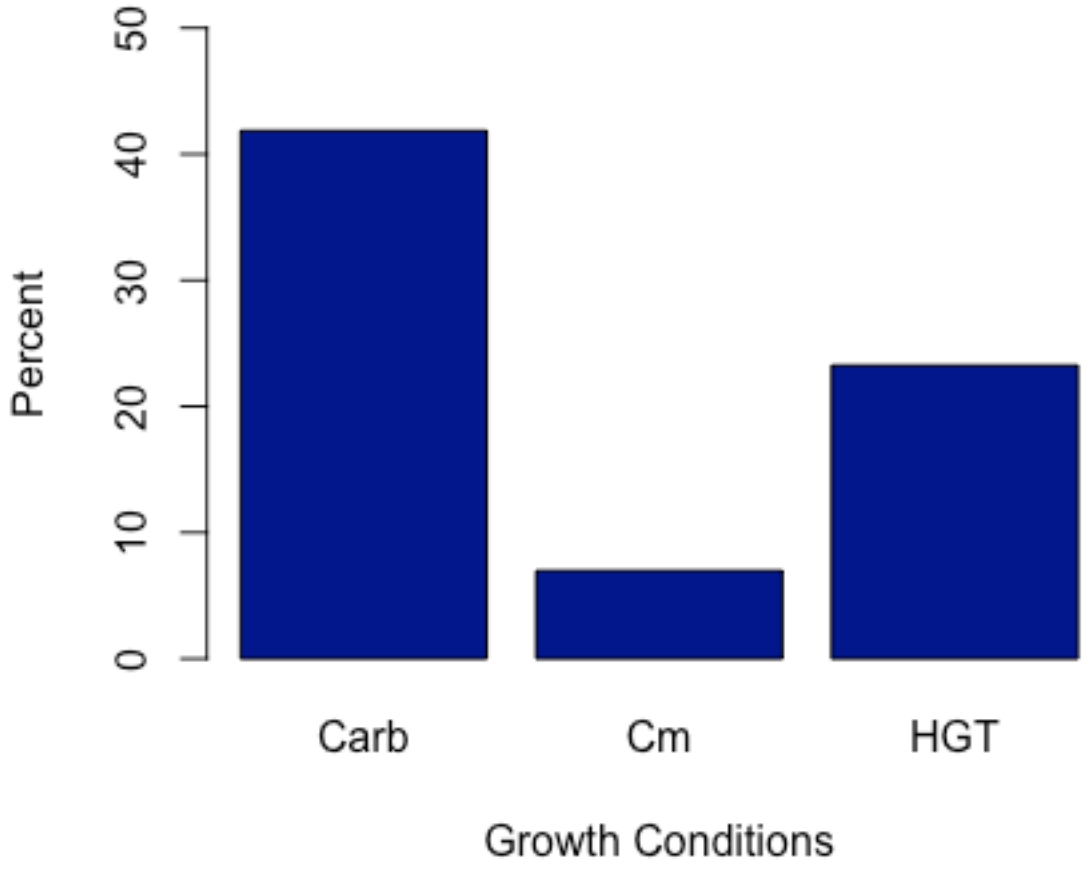

Figure 3: Number of Isolates with Growth in Various Antibiotic Conditions. Each bacterial isolate was cultured in accordance with the strain characterization protocol. Of the 139 strains characterized, 41.9% are Carb resistant, 7% are Cm resistant, and 23.3% of them are capable of HGT. In addition, 55.6% of the Carb resistant strains presented HGT ability. The biggest takeaway is that over half of the strains that are Carb resistant have been found to transfer their resistance to previously susceptible bacteria. This highlights the potential prevalence of HGT throughout the environment in spreading antibiotic resistance.

In addition, the data was split between sampling sites for each growth condition in order to analyze trends between and within locations (Figure 4). Overall, 15 out of 19 sites harbored ESBLs and 11 out of those 15 ESBL sites yielded strains that exhibit HGT ability. In comparison, only 4

sites contained bacteria resistant to Cm. The number of isolates characterized at each site are shown in Table 1.

Table 1: Number of Isolates Characterized by Site

CIEMAS Lab Door	2
CIEMAS Elevator 1	9
CIEMAS Elevator 2	7
CIEMAS Elevator 3	2
CIEMAS Entry Door	15
Divinity Café	12
Chapel	12
West Union	12
Garden Terrace Bench	34
Wilson Gym	8
C1 Bus	3
BC Men's Bathroom	3
BC Women's Bathroom	1
Perkins Printer	5
Medical Center	1
Marketplace	1
Brodie Gym	3
McDonald's Bathroom	4
Perkins Mouse	5

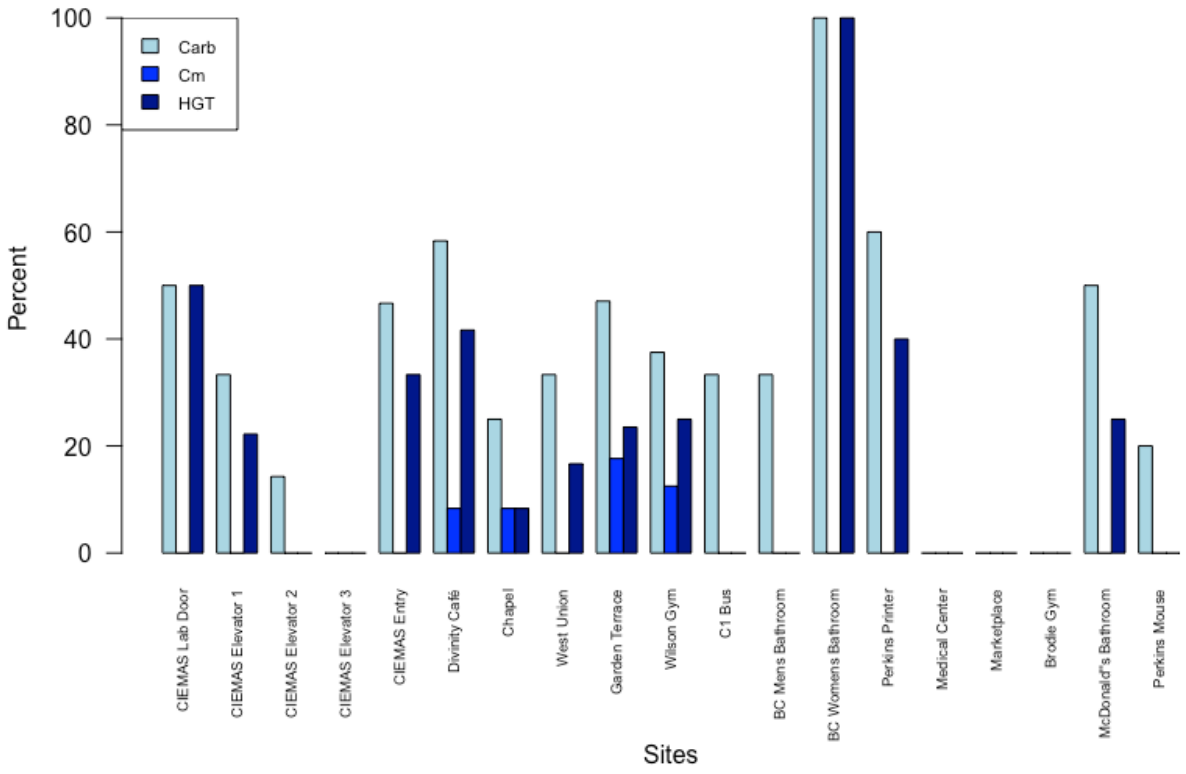

Figure 4: Prevalence of Resistant and HGT Capable Isolates by Location. The most noteworthy sites were the CIEMAS Lab Door (50%), Divinity Café (58.3%), BC Women's Bathroom (100%), Perkins Printer (60%), and McDonald's Bathroom (50%) as at least half of the strains characterized in these locations were identified to be resistant to Carb. In addition, over half of the sites sampled carried bacteria resistant to Carb. With regards to HGT, the most noteworthy sites were the CIEMAS Lab Door (50%) and the door to the BC Women's Bathroom (100%). Understanding where these isolates reside can assist in creating targeted strategies to combat the spread of resistance.

As one of the driving hypotheses for this study centered on finding a trend between the relative abundance of ARB and the proximity of sites to Duke's medical center, some focus was placed on identifying the role of geographic location on ARB prevalence (Figure 5).

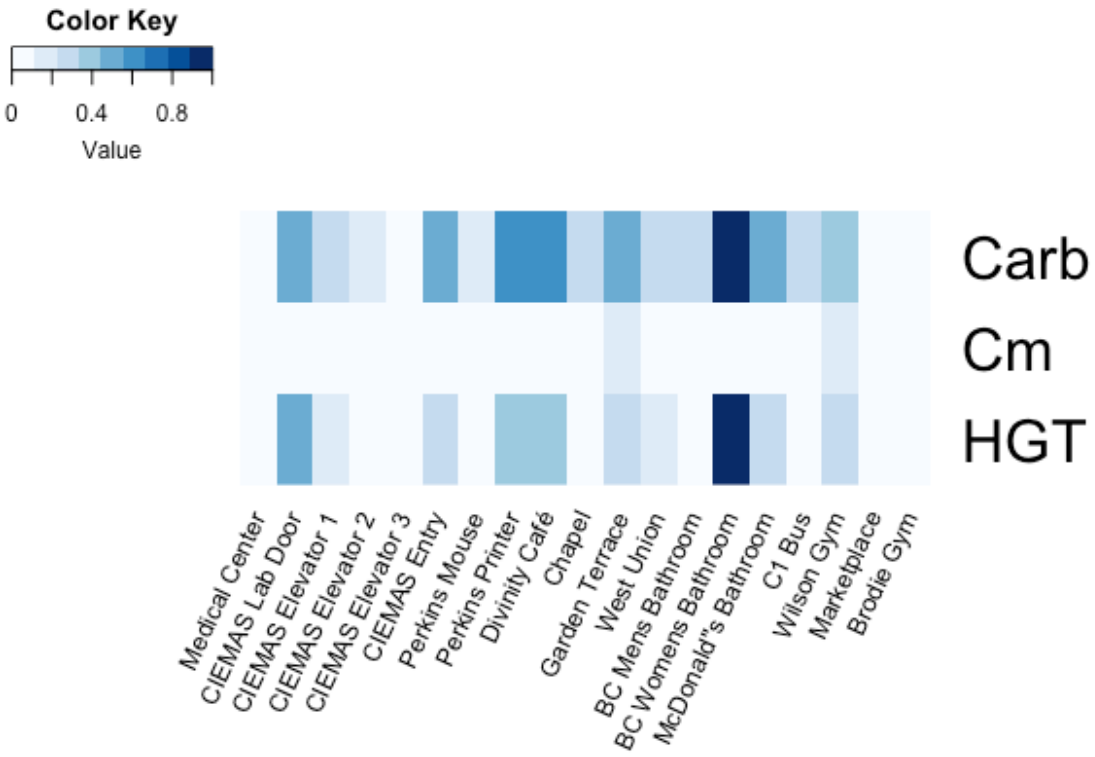

Figure 5: Prevalence of Resistant and HGT Capable Isolates with Respect to the Duke Medical Center. Sites are ordered from their proximity to the medical center, with the leftmost site being the closest and the rightmost being the furthest. The heatmap shows the relative abundance of bacteria that met each of the three conditions (Carb, Cm, HGT). There appears to be no correlation between abundance of resistant bacteria and proximity to the medical center.

Discussion

With antibiotic resistance on the rise, this study aimed to evaluate the potential concerns of horizontal gene transfer in spreading resistance in the environment. Over 700 bacterial isolates that possessed potential clinical relevance (antibiotic resistance and/or enteric species) were collected from Duke University's campus, and 139 of them where characterized. The strain characterization tests focused on identifying resistance to Carb and Cm, and they captured the strains' abilities to transfer resistance to *E. coli*. This study placed an emphasis on HGT because

while previous studies have mapped out a resistome in other environments (Afshinnekoo et al., 2015; Chen H et al., 2019; Chen Z et al., 2019), they have not evaluated whether those resistance genes could be spread by HGT. Understanding the prevalence of HGT in the environment is important to building a fuller picture of how resistance is spread.

In this study, I found that just over half of the Carb resistant isolates were capable of engaging in HGT (Figure 3). These strains are significant as they could confer resistance to other beta-lactam antibiotics like Carb (e.g. ampicillin, vancomycin, carbapenem), which would designate them as ESBLs if they are found to be in the Enterobacteriaceae family of bacteria (Kong et al., 2010). The CDC has classified ESBLs to be a serious threat to human health, causing 26,000 infections, 1,700 deaths, and $40,000 in excess medical costs for each infection each year (CDC, 2013). Evidence that these environmental strains could pass on their resistance genes to *E. coli*, a common ESBL, is of significant concern.

When looking at particular sites on Duke's campus, the CIEMAS Lab Door, Divinity Café, BC Women's Bathroom, Perkins Printer, and McDonald's Bathroom are all noteworthy sites as all had about half or more of the isolates show resistance to Carb (Figure 4). In addition, the majority of the sites that possessed Carb resistant bacteria also displayed evidence of engaging in HGT, with noteworthy sites being the CIEMAS Lab door and the BC Women's Bathroom (Figure 4). The presence of these types of isolates on the CIEMAS Lab door was predicted due to its proximity to the lab that conducts experiments with ESBLs and studies HGT. However, the remaining sites are notable because they are high traffic areas that leave students, faculty, and campus visitors vulnerable to potential antibiotic resistant pathogens. Explanations as to why these particular sites exhibit the highest proportions of ARB are speculative. The McDonald's Bathroom door was likely to harbor enteric species of bacteria due to its proximity to human fecal matter, but it is unclear why sites like the Divinity Café and the Perkins Printer harbor more ARB than

other sites. In addition, there appears to be no correlation between the distance from the Medical Center and the relative abundance of ARB and HGT capabilities, which makes it difficult to pinpoint a possible explanation for what is seen in the data (Figure 5). It is also worth noting that the sample size at each of these locations vary, with the BC Women's Bathroom door only having one data point recorded. This one data point is by no means indicative of the presence of ARB at the location over time. Regardless, efforts should be made to reduce the incidence of these potential pathogens in these specific locations.

The conclusions of this experiment are preliminary, as there needs to be more data to come to a consistent and accurate closure. However, it is already apparent that certain sites were hotspots of hosting ESBLs and bacteria that are HGT capable, making these sites special areas of concern. In addition, the majority of the sites sampled harbored Carb resistant bacteria capable of HGT regardless of their proximity to the medical center, which highlights how prevalent ARB are on Duke's campus. For future directions, it is vital to associate the characterized strains with genomic sequencing data in order to identify the strain, its resistance genes, and its plasmids if present. This is especially important for determining which of the isolates are human pathogens. It would also be important to quantify the efficiency of HGT in several environmental conditions (varying temperatures, moisture, human body conditions, etc.) to gain a better understanding of if these findings translate to what can actually occur in the environment. Finally, work should be done to determine what is driving these trends and observations, and the mechanisms by which they do so. By understanding the mechanisms behind what drives these observations, we can better target methods of combating antibiotic resistance and its spread.

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