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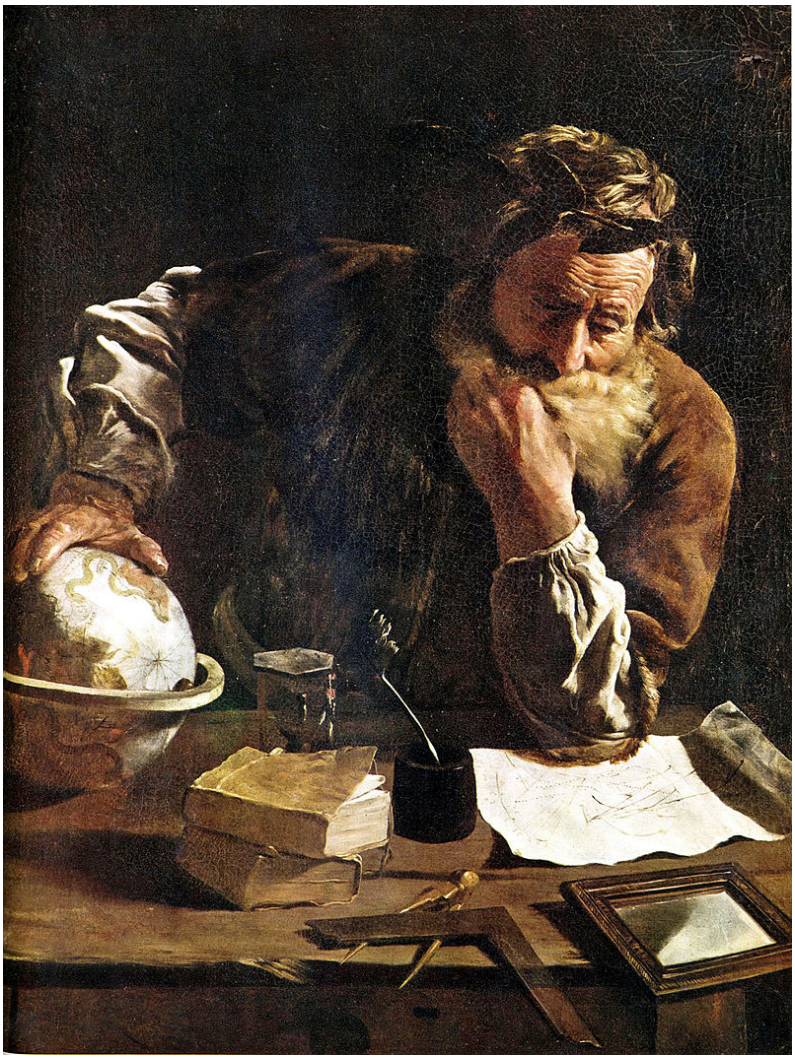

Archimedes Thoughtful by Fetti (1620)

Dedication

This first edition of Eureka is dedicated to my parents, Frank and Patsy Nunez, who instilled in their children a love of the natural world, and nurtured their efforts to explore that world. Both educators with a passion for learning and teaching, they were the inspiration behind creating this publication. Frank and Patsy approach the world in a peaceful, inquisitive way, and I am eternally thankful for their example.

B. Scott Nunez, Ph.D.

To those who reviewed manuscripts for this publication;

Thank you for your time and effort on the behalf of our students. We know the density of your schedule and understand the sacrifice you have made to review our work. This sacrifice is greatly appreciated. The students participating in this research program are enthusiastic, and their work strives to reveal interesting and pertinent things about the world around us. Each manuscript published herein is the result of input from at least three faculty reviewers and the interpretation of this input by the student researchers. We have done our best to address the concerns expressed in each review, and your comments and suggestions have greatly improved the quality of our manuscripts. We will continue to recruit new students, and therefore hope that this is not the last time we call upon you to review such work.

We sincerely thank you for your help,

B. Scott Nunez, Ph.D.

Laura Leverton, Ph.D.

EUREKA!

A Wake Technical Community College Natural Sciences Department Publication of Undergraduate Research

Volume 1, Number 1

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Preliminary examination of the ciprofloxacin resistome in Crabtree Creek, an urban freshwater environment

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Abstract

Ciprofloxacin is a versatile drug that is considered an antibiotic of last resort. Bacterial resistance to ciprofloxacin is increasingly becoming a problem in health care. Maintaining the utility of this important antibiotic depends on how and where resistance is developed and passed on within the microbial community. This study therefore sampled an aquatic environment (Crabtree Creek) within an urban setting (Raleigh, N.C.) to study the abundance and diversity of the ciprofloxacin resistant bacteria. The results of this study indicate a healthy microbial community in Crabtree Creek, one that includes a diversity of ciprofloxacin-resistant bacterial species. Future studies should serially examine a number of sampling sites along Crabtree Creek to further characterize this ciprofloxacin-resistant bacterial population.

Introduction

Ciprofloxacin is a second-generation fluoroquinolone antibiotic that inhibits bacterial reproduction by interfering with DNA replication. Specifically, ciprofloxacin inhibits DNA gyrase, an enzyme that separates bacterial DNA, which allows each strand to be copied, and topoisomerase IV, which helps release replicated sister chromosomes from each other (Drlica and Zhao, 1997). Because it inhibits these critical steps in bacterial reproduction, ciprofloxacin is effective against a broad range of bacteria, including both Gram negative and Gram positive cells. It remains one of the most prescribed antibiotics in the United States, even after recommendations have been made to restrict the use of this class of antibiotics to preserve efficacy (Neuhauser et al., 2003).

Several factors have contributed to the increase in antibiotic resistance in bacteria. In addition to overprescription and inappropriate prescription by physicians, patients may not follow instructions for the proper use of antibiotics. Even worse, the agricultural industry has used antibiotics such as ciprofloxacin prophylactically to treat livestock (CDC, 2013). These practices expose bacteria to sublethal doses of antibiotic, selecting for bacteria that are less susceptible to such antibiotics (reviews on the causes and problems associated with antibiotic resistance can be found at: Antibiotic/Antimicrobial Resistance, 2016; Davies and Davies, 2010). Therefore, despite the recent recommendations against the overuse of ciprofloxacin, bacterial resistance to ciprofloxacin is becoming a major problem.

Many bacterial strains become resistant to ciprofloxacin through mutations in DNA gyrase and topoisomerase IV, rendering these enzymes insensitive to ciprofloxacin (Jacoby, 2005). Other strains have evolved proteins (e.g., QNR proteins) that interfere with the ability of ciprofloxacin to interact with DNA gyrase. Some strains carry mutant efflux pumps that are able to pump ciprofloxacin out of the cell before it can inhibit their targets. Finally, there are strains that have enzymes which can chemically modify ciprofloxacin, rendering the drug ineffective (mechanisms of ciprofloxacin resistance are reviewed extensively in Li, 2005; Jacoby, 2005; Strahilevitz et al., 2009 and Jacoby et al., 2014; among others).

There are over 20,000 different bacterial genes associated with antibiotic resistance (Liu and Pop, 2009). Perhaps even more concerning is the ability of many bacteria to transfer genetic information (and therefore possibly antibiotic resistance) to naïve species. There is evidence for a positive correlation between the abundance of antibiotic-resistant bacteria and

human activity (Keen and Patrick, 2013). This may be especially true in aquatic ecosystems, which can receive input from a number of diverse and sometimes distant sources. Aquatic ecosystems can also serve to distribute antibiotic resistant bacteria (ARB) to new, naïve ecosystems. Monitoring environments that are heavily impacted by humans will allow for early detection of increases in antibiotic resistant bacteria and potentially the detection of new antibiotic resistance genes (Ashbolt et al., 2013). Therefore, the objective of this study was to examine the abundance of ciprofloxacin resistant bacteria (CRB) in Crabtree Creek, a freshwater stream that flows through the city of Raleigh, North Carolina, a city with a population of approximately 440,000 people (Raleigh Demographics, 2017). A sample was taken from Crabtree Creek in an area that has high human traffic, and is also frequented by pets and wildlife. This sample was then used to inoculate a general medium containing ciprofloxacin to determine the abundance of CRB in these water samples. The results of this study indicate that there is a population of CRB at this site. Further research must be conducted in order to determine how stable and extensive the population is within the creek, the potential sources of this population, and if such resistance is encoded by mobile elements.

Materials and Methods

Collection, transport and storage of water samples

This study examined the antibiotic resistance within Crabtree Creek, a fairly large body of water whose headwaters originate in north Raleigh (an area of low human activity), and travels through the city of Raleigh, eventually draining into the Neuse River in eastern Raleigh (figure 1). In an initial study, samples were collected from Crabtree Creek near Raleigh

Figure 1: Map of Raleigh, North Carolina. The bright, red line indicates the approximate path of Crabtree Creek and the red dot indicates the sampling site. Map courtesy of https://maps.raleighnc.gov/iMAPS/index.html

Boulevard. In addition to a bike trail and walkway, the area is populated with turtles and ducks that appeared to be well adapted to the presence of humans.

A sampling site near the edge of the stream near the part of the walkway that crosses the creek was chosen. This allowed easy access for our sampling, and a good landmark to identify the site for future sampling. The sample was collected on September 16, 2014 at 11:30am. Using a sterile, plastic bulb pipet, approximately 10 ml of water was aseptically transferred from the surface of Crabtree Creek to a sterile, screw-top tube. The cap was secured and the tube placed in a container containing frozen ice packs to keep the sample cool

and was then transported to the microbiology lab at the Perry Health Sciences campus of Wake Technical Community College.

Inoculation and growth of samples

Once arriving at the laboratory, the sample was tested for ciprofloxacin resistance by inoculating two different types of media. The first media was tryptic soy agar (TSA), a standard general growth media. These plates provided an indication of the abundance and diversity of the total microbial flora in our samples. The second media was TSA to which ciprofloxacin was added at a final concentration of 100 µg/ml (TSA+C). Each agar plate was inoculated with 100 µl of water sample. A sterile glass rod bent at a 90 degree angle was used to spread the sample over the surface of the agar. This procedure was repeated three times for each type of TSA plate, so that the water sample was used to inoculate six plates total (three TSA and three TSA+C plates). All inoculated plates were incubated at room temperature for approximately 24 hours before being examined. The plates were then stored in the refrigerator until they could be counted.

Examination and enumeration of microbial flora from Crabtree creek

Because there were significantly greater numbers of colonies on TSA plates than on TSA+C plates, the number of colonies on each TSA plate was estimated by drawing 20 cm circles at random on the back of the plates. The colonies in these circles were counted and the number of colonies per square centimeter was calculated, and then used to extrapolate the total number of colonies on the plate. Because there were fewer colonies on the TSA+C plates, the total number of colonies on these plates were simply counted. All plates were counted by

two investigators, and the average number of colonies from each count was obtained and recorded.

Results

The TSA plates revealed an abundance of diverse microorganisms (figure 2). Colonies ranged in size from very small (~1 mm) to moderate (~7 mm). Colonies were mostly white to grey, but there were some small orange and yellow colonies as well. Several colonies were rhizoid or filamentous.

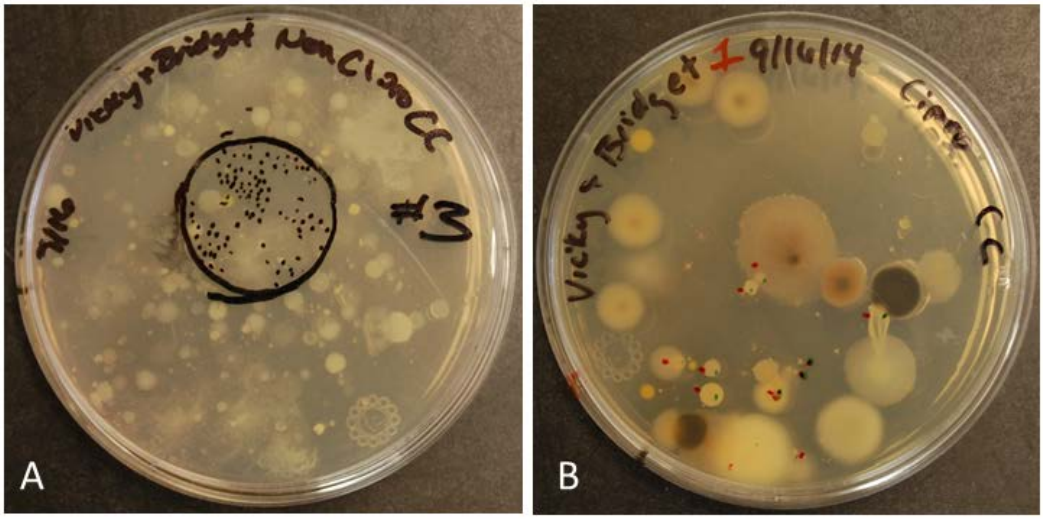

Figure 2: Representative images of (A) growth of Crabtree Creek water on tryptic soy agar (TSA) and (B) TSA plus ciprofloxacin. Both media yielded robust growth of a variety of microorganisms.

The TSA+C plates had fewer colonies, but the range of size was greater, although colonies were larger in size on average than seen on the TSA plate. There were more pigmented colonies as well, and a higher percentage was rhizoid or filamentous.

The large rhizoid or filamentous colonies (usually also a darker color) on the TSA and TSA+C plates were assumed to be fungi. They were therefore not counted in the subsequent colony counts. The average number of bacterial colonies on the TSA plates (as determined independently by two investigators) was 226 (figure 3). The average number of bacterial colonies on the TSA+C plates (as determined independently by two investigators) was 10.

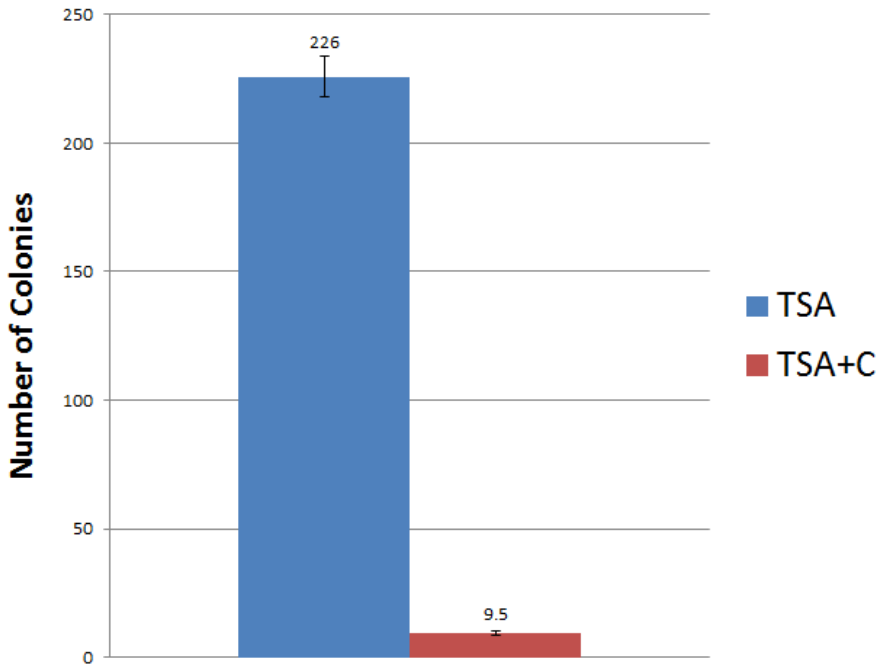

Figure 3: Number of bacterial colonies on TSA versus TSA plus ciprofloxacin.

Conclusions

The results of this study support the hypothesis that local aquatic environments harbor CRB. Given the various possible inputs of bacteria into Crabtree Creek (e.g., humans, pets, wildlife, businesses) it was expected that a diverse abundance of bacterial colonies would grow on a general medium (TSA). A significant number of fungal cells grew on TSA and TSA+C. However, there were proportionally more fungal colonies on the TSA+C plate, probably because

of the release of competition through the elimination of ciprofloxacin susceptible bacteria on the TSA+C plate. Future studies should include an antimycotic such as clotrimazole to reduce fungal growth. The results of this study indicate a robust microbial community in Crabtree Creek at the sample site.

Given the concentration of ciprofloxacin used (100 μg/ml; 20 times the concentration normally used for environmental monitoring), it was surprising to see the level of CRB on the TSA+C medium. This concentration of ciprofloxacin was used to eliminate any moderately resistant species, and to isolate any highly resistant species. Although safety precautions precluded the identification the bacterial species from these plates, there were at least five distinct types of colonies found on the TSA+C media, indicating the presence of several bacterial species in this aquatic environment that are highly resistant to ciprofloxacin.

The presence of highly resistant bacteria is concerning and may represent a public health threat. Such bacteria may infect individuals who come into contact with Crabtree Creek water, and such infections might be more difficult to treat. On another level, these bacteria may be able to transfer ciprofloxacin resistance to other bacterial species, through mobile elements such as plasmids. Future studies should attempt to identify specific species of highly ciprofloxacin-resistant bacteria. Once identified, the specific mode of resistance could be determined, and perhaps the gene(s) responsible for ciprofloxacin resistance discovered and whether these genes are found on mobile elements.

Crabtree Creek offers a superb model for the study of ABR in aquatic environments. It is a well-defined stream that originates from Lake Crabtree in Cary, N.C., travels north through

William B. Umstead Park and then southwest through north Raleigh. It terminates at the Neuse River in southeast Raleigh. The creek passes by and through a variety of urban environments, including parks, commercial properties, residential areas, sewage treatment plants and hospitals. Therefore, there are a variety of potential sources which could impact the CRB community of this environment. There is an extensive greenway that follows much of the creek as it passes through Raleigh. This greenway allows easy access to multiple sampling sites and the opportunity for humans to further impact the microbial community of the creek. A future study might examine how the CRB community changes along the length of Crabtree Creek, and whether there are spatial or temporal hotspots regarding such bacteria. Such studies would greatly improve our understanding of the dynamics of CRB communities, and serve to monitor aquatic environments for changes in the community that might threaten human health.

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An assessment of antibiotic resistant bacteria in the whitetail deer population at the Durant Nature Preserve in Raleigh, NC

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Abstract

Human-assisted and natural processes have contributed to a growing number of resistant bacterial species, rendering a variety of antibiotics useless and some bacterial infections untreatable. While extensive research has been conducted on the distribution of resistant bacteria in agriculture and health care, only a few studies have focused on the spread of antimicrobial resistance by wildlife. This study examines the prevalence of Ciprofloxacin resistant bacteria in a population of whitetail deer at the Durant Nature Preserve in Raleigh, N.C. Five samples of deer scat were collected in the woods of the park and subsequently cultured on agar, with or without Ciprofloxacin (25 μg/ml). Two samples of Durant Nature Preserve Lake Number 1 water were obtained and examined in the same manner. One of the scat samples yielded bacteria that displayed resistance to Ciprofloxacin, adding to the growing evidence that wildlife may be an important vector of AMR.

Introduction

Antimicrobial resistance (AMR) is one of the most urgent public health issues of our time. In hospitals, AMR can cause a patient to remain contagious for longer periods, increasing the possibility of spreading resistant microorganisms. Some bacterial strains are resistant to all antibiotics, and patients infected with such bacteria face dire consequences such as amputation and death. AMR therefore increases the need for more extensive treatments, promotes longer hospital stays and increases the probability of mortality, while raising health care costs for families and society in the process (About, 2015).

Intrinsic natural AMR can occur in several ways. The bacterial cell may simply lack a target site or transport system necessary for the function of an antibiotic (Bacterial, 2011). The antibiotic may not have access to its intended target. For example, the cell envelope in Gram-negative bacteria is composed of an outer membrane, a cell wall and a cytoplasmic membrane;

these layers may inhibit the penetration of antibiotics into the cell. So, some bacteria are resistant to some antibiotics simply because those antibiotics were not designed to target them.

However, some bacteria that were once susceptible to antibiotics are now resistant to those same antibiotics. Several natural processes lead to new strains of AMR bacteria. Bacteria have adapted efflux pumps as reverse transport mechanisms that transport an antibiotic out of the cell. Also, bacterial enzymes may either chemically modify an antibiotic and render it inactive or degrade the drug, causing it to become ineffective (Bacterial, 2011). Further, mutations in the bacterial genome can render once susceptible targets such as ribosomes and enzymes less vulnerable to the effects of antibiotics. The individual carrying the mutation passes it to the next generation, which is also resistant. Through natural selection, non-resistant members die out, while the resistant members survive. This process is referred to as vertical transfer (Bacterial, 2011).

However, bacteria can also transfer resistance genes to other, sometimes unrelated, bacteria through horizontal gene transfer. During horizontal gene transfer, small pieces of DNA (transposons, integrons and plasmids) can serve as vectors that carry genes (and sometimes AMR genes) between neighboring bacteria (Antimicrobial, 2011). Therefore, bacteria have a mechanism by which they can become resistant to antibiotics to which they have never been exposed, making it extremely important that humans do not contribute to the increase in AMR genes that can be transferred.

The use of antibiotics is the single most important cause of the development of antibiotic resistance worldwide (About, 2015). Resistance can be categorized into human-assisted and natural. Human-assisted resistance is centered around the overuse and overprescription of antibiotics. Nearly half of antibiotics are prescribed unnecessarily (i.e. against viral infections which will not respond to antibiotics) or in the wrong dosage (About, 2015). Physicians may also prescribe a broad-spectrum antibiotic instead of a more targeted antibiotic for a specific infection. A patient may stop taking the drug too early or may skip doses, allowing resistant

bacteria to survive. These resistant bacteria can then be transmitted to other people or the environment.

Agriculture may also be another major cause of the spread of AMR. For example, *Salmonella* and *Campylobacter* species can become resistant when antibiotics are administered to common food animals. Through unsafe handling and food preparation, these bacteria enter and survive in the human gut, and individuals may spread the germs within their community. One study comparing antibiotic resistance across a variety of different species found that the highest levels of resistant *Escherichia coli* isolates in farm environments occurred in swine and poultry, since antibiotics are added to their feed to promote growth and prevent disease (Sayah et al., 2005).

Farm animals may not be the only animals that can serve as a source of AMR. Interestingly, some studies conducted on bacterial resistance in wildlife indicate a low but significant resistance across several species. A study conducted on fecal samples of wild boars in Portugal found extended-spectrum beta-lactamase (ESBL)-producing *E. coli* in eight of seventy-seven samples (Poeta et al., 2009). Another study on Iberian wolves reported that nearly two-thirds of *E. coli* isolates were resistant to one or more of a total of 19 antimicrobials tested (Simoes et al., 2012). The authors speculate that the wolves acquired the resistant bacteria through their prey, which mostly consists of livestock from farms and carcasses improperly disposed of by slaughterhouses (Simoes et al., 2012). Therefore, wild animals could serve as both reservoirs and vectors of AMR.

Ciprofloxacin is one of the most important and powerful antibiotics available to medical professionals. Ciprofloxacin is a broad-spectrum fluoroquinolone antibiotic that has been used to treat serious and resistant infections since the late 1980's. The antibiotic was derived from the synthetic quinolone family by replacing the eighth carbon atom of the backbone with a nitrogen atom and adding a fluorine atom at the sixth position, thus making it more potent and able to provide broad-spectrum protection against a variety of Gram-positive and Gram-negative bacteria (Ball, 2000). Fluoroquinolones like Ciprofloxacin are powerful inhibitors of bacterial type II topoisomerases, a class of enzymes that help prepare DNA for replication and

transcription (Drlica, 1999). Specifically, fluoroquinolones target DNA gyrase in Gram-negative bacteria and topoisomerase IV in Gram-positive bacteria, (Drlica, 1999; Drlica et al., 2008; Drlica et al., 2009). This leads to impaired DNA replication at lower concentrations and cell death at lethal concentrations (Drlica, 1999; Drlica et al., 2009).

Due to the popularity and wide-spread application of fluoroquinolones, various microorganisms have developed resistance mechanisms to the drug. Target-site mutations in genes that encode type II topoisomerases are the most common mechanism of fluoroquinolone resistance. The regions on the genes where these mutations arise are called the quinolone resistance-determining region (QRDR) (Yoshida et al., 1990; Yoshida et al., 1991). Mutations in the QRDR region of these genes result in amino acid substitutions, thus altering the target protein structure and consequently the fluoroquinolone-binding affinity of the enzyme, which leads to drug resistance (Hooper, 2000; Piddock, 1999).

Besides target-site mutations, there are transmissible quinolone-resistance mechanisms, which are often encoded on plasmids and are referred to as plasmid-mediated quinolone resistance (PMQR) genes (Martinez-Martinez et al., 1998). The original PMQR gene, qnr, was first discovered on a plasmid of a clinical isolate of *Klebsiella pneumoniae* (Martinez-Martinez et al., 1998). The structure of a qnr protein suggests that fluoroquinolone resistance is attained by binding of the qnr protein to topoisomerase, which physically prevents the linking of the antibiotic with the target enzyme (Xiong et al., 2011). A different PMQR gene encodes a variant of an aminoglycoside acetyl transferase, ααc(6')-lb-cr, which has the ability to degrade some fluoroquinolones (Martinez-Martinez et al., 1998). This gene is also commonly found on plasmids and inhibits the susceptibility to Ciprofloxacin and Norfloxacin by acetylating the amino nitrogen compound on the piperazinyl substituent present in these drugs (Robicsek et al., 2006). Another group of fluoroquinolone-resistance genes comprise the oqxAB and qepA efflux systems, which encode transporters that export fluoroquinolone molecules out of the cell (Robicsek et al., 2006). Besides plasmid transport systems, chromosomal efflux pumps are able to actively rid fluoroquinolones from the bacterium. These pumps belong to various classes, for example the major facilitator superfamily (MFS) pump NorA of *Staphylococcus aureus* and the resistance nodulation division (RND) family of tripartite transporters of Gram-negative

pathogens (Kaatz and Seo, 1995; Piddock, 2006). Efflux systems are essential for the development of high-level fluoroquinolone resistance because mutated target-site genes no longer cause clinical resistance once these systems are inactivated (Oethinger et al., 2000; Ricci et al., 2006; Ricci et al., 2004.)

There are very few studies examining the prevalence of Ciprofloxacin-resistant bacteria in wildlife. Therefore, the goal of this study was to determine whether whitetail deer (*Odocoileus virginianus*) in Durant Nature Preserve harbor Ciprofloxacin-resistant bacteria and could therefore serve as vectors of AMR. Durant nature preserve is a 237-acre nature park located in a heavily populated area of North Raleigh. The park is surrounded by private residences and a large logistics and warehouse complex. Five miles of hiking and mountain biking trails, two lakes open for fishing, two tennis courts and three picnic shelters create a popular place for residents to spend their leisure time (Durant, 2016). Therefore, the park is ideal for the study of the interactions and possible transfer of antibiotic resistant bacteria between humans and wildlife. According to the park manager, the deer population was never officially measured; however, whitetail deer are numerous and commonplace (Corbally, 2016). They tend to migrate, and fawns are regularly born in the park each year. However, the deer population is not controlled by the park administration at this time. Due to the parks' popularity, the deer have almost lost their fear of humans (Corbally, 2016). The deer may acquire resistant microorganisms by coming in contact with feces from other animals, in particular pets, or through exposure to feces in bodies of water. Our findings indicate that deer can indeed harbor Ciprofloxacin-resistant bacteria.

Materials and Methods

Powdered tryptic soy agar (TSA; Carolina Biological Supply item number 788421) and MacConkey agar (Carolina Biological Supply item number 784481) were prepared as per manufacturer's instructions, with the exception that Clotrimazole (Sigma-Aldrich item number C6019) in ethanol was added to agar at a final concentration of 10 μg/ml in all plates to inhibit the growth of fungi. Agar plates containing Ciprofloxacin (Fluka item number 17850-5G-F) were prepared by adding Ciprofloxacin dissolved in 0.1N hydrochloric acid. Control plates (i.e. plates

with no Ciprofloxacin) received a volume of 0.1N HCL equal to that used in the Ciprofloxacin plates. Clotrimazole, Ciprofloxacin and hydrochloric acid were added to molten agar only after allowing the agar to cool to 55 ^{o}C.

Samples of deer scat were aseptically collected from Durant Park using either sterile forceps or sterile tongue depressors and then transferred into sterile urine collection cups. Care was taken to avoid dirt or other material that might contaminate the scat. The samples were stored on ice until transferred to the laboratory. Specifically, on March 10, two samples (DP1 and DP2) were collected as described above from a heavily wooded area of Durant Park between 4:00 PM and 6:00 PM. The samples were transported to Wake Technical Community College Health Science Campus (PHSC) and extracted with 1 ml PBS. Using the spread plate method, aliquots (100 µl) were plated on TSA plates containing 25 µg/ml Ciprofloxacin and 5 µg/ml Clotrimazole at 7:00 PM and incubated overnight at 37 ^{o}C. In addition, control plates with no Ciprofloxacin and 5 µg/ml Clotrimazole were prepared from the same two samples using the same technique and incubation method.

A second set of three samples (DP 3-5) was collected on March 12 between 4:00 – 6:00 PM. The samples were stored on ice until they were transported to PHSC and plated as described above.

To determine if the water in one of the lakes of Durant Park could serve as a reservoir for Ciprofloxacin resistant bacteria, water samples from Camp Durant Lake Number One were aseptically collected on April 10 (DP 6). A final sample, DP 7, was collected in the woods on April 22 and plated on two TSA plates containing 10 µg/ml Ciprofloxacin and 10 µg/ml Clotrimazole and two MacConkey agar plates containing 10 µg/ml Ciprofloxacin and 10 µg/ml Clotrimazole. Samples were also plated on agar containing only Clotrimazole. All samples were incubated overnight at 37 ^{o}C.

Results

Aliquots of all samples yielded robust growth on media that did not include Ciprofloxacin (For example see Figure 1). Most of the Ciprofloxacin-containing agar plates showed no bacterial growth, although there were several fungal colonies despite the presence

of Clotrimazole. However, sample DP3 harbored bacteria resistant to Ciprofloxacin. These Ciprofloxacin-resistant bacteria grew in either light pink or cream colored round colonies.

The DP 7 sample from the Camp Durant Lake Number One plates was inoculated on both TSA and MacConkey agar. This sample yielded good growth on both TSA and MacConkey agar with no Ciprofloxacin, but yielded no growth when Ciprofloxacin was added.

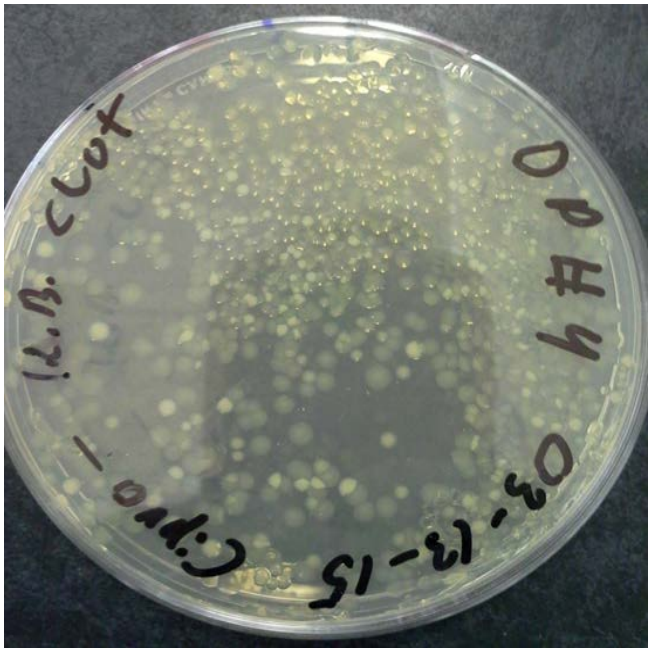

Figure 1: Example of growth from Durant Park samples plated on medium without Ciprofloxacin. All samples yielded colonies that were too numerous to count on this medium.

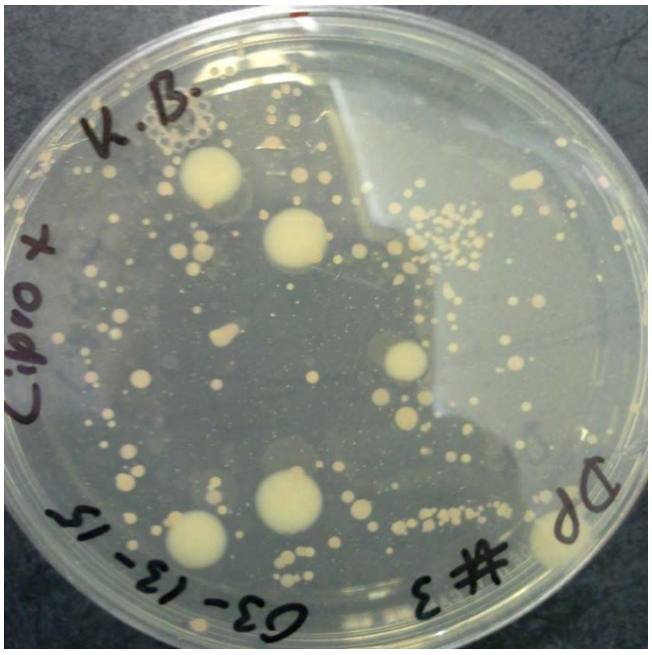

Figure 2: Growth from Durant Park sample DP3 plated on medium with Ciprofloxacin. This sample yielded two different types of small, round bacterial colonies, one a cream color and one a pinkish color. The larger colonies here are likely fungi.

Conclusions

Many wildlife species play an important role as vectors of AMR, especially when human population growth encroaches on their habitat. Deer move from one wooded area to the next and can often be seen close to suburbs, where they can spread resistant bacteria. This study shows that whitetail deer may carry bacteria resistant to Ciprofloxacin and contributes to the growing number of research emphasizing the significance of wild species as carriers of resistant organisms. However, our study had some limitations due to practical constraints. First, only one relatively small park was investigated. The exact number of deer roaming the park during sampling is not known, and the number may not have been statistically significant. Consequently, the samples could have been produced by very few if not the same animal. In order to improve the results in further studies, more diverse environments such as parks, woods and suburban areas should be examined to ensure a larger and scientifically more significant sample size. Nevertheless, the fact that antibiotic resistance was found even in this small study suggests the need for further studies on the spread of resistant organisms by a variety of wildlife.

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Stability of bacterial biochemical characteristics following freeze-storage

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Abstract

Freezer stocks are a convenient way to store microorganisms, but freezing could serve as a selection pressure, creating a population with different characteristics than the parental population. To examine this possibility, freezer stocks of three species (*Staphylococcus aureus, Bacillus cereus*, and *Escherichia coli*) were created. The stability of several physiological characteristics (hemolysis, citrate transport, carbohydrate fermentation, hydrogen sulfide production, urea degradation and antibiotic resistance) within these freezer stocks were then examined over a five week period. The results indicate that, in all three species, most physiological characteristics were stable after freezing. Interestingly, the freeze-thaw cycle induced a gene (urease) present in *Staphylococcus aureus,* but not expressed in these cells at the beginning of this experiment. Investigators must therefore be careful not to confuse traits induced by physiological challenges with new traits created by selection through the freeze-thaw cycle.

Introduction

Creating freezer stocks is an easy and efficient way for long-term storage of microorganisms. However, not all bacteria will survive the cycle of freezing and thawing; some cells will be more freeze-tolerant than others (Harrison, 1955). Consequently, the creation and subsequent thawing of bacterial freezer stocks may select for a new population of cells that differ from the parent population in freeze tolerance, and perhaps other characteristics.

The conservation of bacterial cultures is critical to several areas of study. Bacteria are used in a number of scientific fields, including ecology, physiology, biochemistry, genetics, and medicine. In education, bacterial stocks allow instructors to use the same bacteria each semester without having to purchase new stocks or become familiar with new strains. Food

industries keep frozen stocks of microorganisms. Yeast and bacteria, for example, are used to produce foods such as cheese, beer, wine and sauerkraut. Industries keep frozen stocks of microorganisms to use in these processes, and changes in the metabolic profiles of these microorganisms because of freeze storage could affect the flavor and quality of these foods. For example, Alrabadi (2015) concluded that freezing milk for long periods could lead to low quality cheese due to changes in the bacterial population.

The selection of bacteria through freeze-thaw cycles has important universal implications to all fields that utilize freezer stocks. For example, bacteria could show variance in biochemical and physiological characteristics that are used to identify them. Characteristics such as lactose fermentation, citrate transport, and the metabolism of urea are all common tests used to identify bacteria. Shifts in such characteristics could therefore make it more difficult for instructors to teach students how to identify certain bacterial species using these tests. Such changes could also potentially alter the results of scientific studies. Perhaps most importantly, bacterial strains used to create stocks could emerge from the freezer with distinct virulence characteristics (e.g. hemolysins; antibiotic resistance) and alter pathogenic efficiency. Bacteria can gain antibiotic resistance rapidly. In some European countries, the prevalence of antimicrobial resistance in *Staphylococcus aureus* strains is very high, up to 50% (O'Neill, 2014). It is possible that freeze-selection could create strains that are more resistant to antibiotics, and therefore harder to treat. The unintended creation of new antibiotic resistance strains through the creation of freezer stocks would add to this already pressing problem. This study used a suite of basic microbiological assays to test the hypothesis that basic bacterial characteristics

such as hemolysis, carbohydrate fermentation, citrate transport, urea metabolism and antibiotic resistance will not change after freeze-thaw treatment.

Materials and Methods

Three different bacterial species were purchased from Carolina Biological Supply to be used in this study: *Bacillus cereus* (item number 154870A), *Escherichia coli* (item number 155070A), and *Staphylococcus aureus* (item number 1555554A). These lyophilized strains were rehydrated in the provided medium and aliquots used to inoculate sheep's blood agar. Isolated colonies from each plate were used to inoculate tryptic soy broth (TSB; Difco item number 290613). These overnight cultures were then used to inoculate an initial set of media as well as to create a freezer stock using the "Microbank" system (Pro-Lab Diagnostics), which uses a glycerol solution (final concentration of 15%) as a cryopreservative. After a week, a sterile needle was used to pick up one bead and inoculate TSB to revive the frozen samples. This was repeated once a week for the duration of the five week study.

Inoculation of media

Sheep's blood agar (SBA) (Becton Dickinson item number 221261) is a differential media containing general nutrients and 5% sheep's red blood cells. Blood agar is used to detect hemolysis, of which there are three basic types. β-hemolysis is the complete lysis of erythrocytes, and is shown as a clear area around the colony. α-hemolysis is the partial lysis of erythrocytes, shown as a greenish to brownish discoloration of medium. γ-hemolysis is simple growth with no lysis of erythrocytes. In this study SBA was inoculated with the quadrant streak method.

Mannitol salt agar (MSA; Becton Dickinson item number 221173) contains 7.5% sodium chloride, which selects for the growth of halophilic or halotolerant bacteria (i.e. bacteria that can grow in high concentrations of salt). The carbohydrate mannitol and the pH indicator phenol red are also added in order to differentiate between bacteria that can use mannitol in acid fermentation, and those that cannot (Leboffe, 2008). MSA was inoculated with the quadrant streak method.

MacConkey Agar (MAC; Carolina Biological Supply item number 82-1682) contains bile salts and crystal violet as selective ingredients, as well as neutral red (a pH indicator) and lactose, to differentiate between lactose fermenters and nonfermenters. MacConkey agar is used to select for the growth of Gram negative enteric bacteria (Leboffe, 2008). MAC was inoculated with the quadrant streak method.

Triple sugar iron agar (TSI; Carolina Biological Supply item number 82-7442) contains glucose, sucrose and lactose with phenol red as a pH indicator. Bacteria can produce acidic and gaseous products when fermenting sugars. The production of acid during fermentation will change the phenol red in TSI from red to yellow. Gas is seen as bubbles trapped in the solid medium, or as cracks formed when gas pressure has separated the medium. Some bacteria can reduce sulfur, producing hydrogen sulfide gas. The iron in TSI will react with this gas to form a black precipitate. TSI, therefore, differentiates bacteria based on their ability to ferment carbohydrates and/or produce H_2S (Leboffe, 2008). This medium was prepared as a slant and inoculated using a stab and streak technique.

Simmons' citrate agar (Becton Dickinson item number 221026) contains sodium citrate, ammonium ions, and bromothymol blue dye which acts as a pH indicator. Citrate agar tests an organism's ability to transport citrate into the cell and use it as a carbon source. Citrate permease is the enzyme generally used in this process. At a pH of 7, the bromothymol blue appears green. Bacterial transport and use of citrate creates alkaline products, thus increasing the pH of the medium and changing the color to blue (Leboffe, 2008). Simmons' citrate agar was prepared as a slant and inoculated using a simple streak technique.

Urea agar (Carolina Biological Supply item number 82-7462) contains urea and uses phenol red as pH indicator. Urea agar is used to identify organisms that have urease, an enzyme that hydrolyzes urea to ammonia and carbon dioxide (Leboffe, 2008). This medium was prepared as a slant and inoculated using a simple streak technique.

The Kirby Bauer Assay measures sensitivity of bacteria to antibiotics. After creating a bacterial lawn on Mueller-Hinton agar (Becton Dickinson item number 221800), antibiotic disks were evenly placed on the surface of the agar using sterile forceps. All the disks were dropped carefully above the agar to avoid touching the surface with the forceps. The antibiotics tested were purchased from Carolina Biological Supply and listed in Table 1. All samples were incubated overnight for 20-24 hours at 37° C in ambient atmosphere.

Table 1: Product information for the antibiotics used in this study.

Antibiotic	Manufacturer	Vendor	Item Number	Disk abbreviation	Concentration per disk
Augmentin (Amoxicillin + Clavulanic acid)	BBL	Carolina Biological Supply	806014	AMC	20 micrograms and 10 micrograms respectively
Bacitracin	Oxoid	Fisher	806022	B	10 micrograms
Chloramphenicol	BBL	Carolina Biological Supply	806058	C	30 micrograms
Nalidixic Acid	Oxoid	Fisher	CT0031B	NA	30 micrograms
Novobiocin	BBL	Carolina Biological Supply	806492	NB	30 micrograms
Optochin	Oxoid	Fisher	DD0001B	OP	5 micrograms
Penicillin	BBL	Carolina Biological Supply	806296	P	10 units
Polymyxin	Oxoid	Fisher	CT0044B	PB	300 units
Tetracycline	BBL	Carolina Biological Supply	806476	TE	30 micrograms
Vancomycin	BBL	Carolina Biological Supply	806492	VA	30 micrograms

Experimental plan

Once freezer stocks were established, the same experimental plan was followed every week for a total of five weeks. On the first day, liquid cultures were started by aseptically removing a single bead from each freezer stock and placing it into individual tubes of TSB. On the second day, following overnight growth, the TSB culture was used to inoculate SBA, urea agar, TSI agar, Simmon's citrate agar and Muller-Hinton agar. On the third day, the media was examined for growth and any changes in physiological or biochemical characteristics of the bacteria, as indicated by the media inoculated.

At week 5, *S. aureus* was positive for urease, unlike the previous four weeks. In order to confirm the bacteria's identity and rule out contamination, several assays were conducted. A

gram stain was used to confirm shape, arrangement and gram state. MSA was inoculated to differentiate *S. aureus* (a mannitol fermenter) from other *Staphylococcus* species. MacConkey agar was used to eliminate possible contamination with urease-positive enteric Gram negative rods. Finally, the Kirby-Bauer plates from week 1 through week 5 were used as the source of inoculum for new urea agar cultures.

Results

Sheep's blood agar

Both *S. aureus* and *B. cereus* displayed prominent zones of clearing around bacterial colonies, indicating β-hemolysis. *E. coli* cultures were γ-hemolytic, as there were no indications of hemolysis. These hemolytic properties did not change over the course of the study.

Triple sugar iron agar

After 24 hours, TSI media inoculated with *B. cereus, S. aureus,* and *E. coli* were completely yellow, indicating these species could use sucrose, lactose, or both sugars in acid fermentation. However, gas bubbles were not seen and hydrogen sulfide was not produced. There were no changes in these results during the five weeks.

Simmon's citrate agar

There was no growth and the medium remained green for all three species with this test, indicating they were not able to transport citrate. The ability to transport citrate did not change over the five weeks.

Urea agar

During the first four weeks, *B. cereus, E. coli,* and *S. aureus* were all urease negative, as indicated by a lack of color change in the agar. However, the urea agar which was inoculated on week 5 with *S. aureus* turned pink, indicating the presence of urease in *S. aureus. B. cereus* and *E. coli* remained urease negative for the entire five week experiment.

Kirby-Bauer assay

B. cereus was susceptible to Chloramphenicol, Vancomycin, Tetracycline, Nalidixic acid, and Novobiocin and resistant to Penicillin, Bacitracin, Polymyxin, Augmentin, and Optochin (Table 2).

Table 2: Susceptibility of *Bacillus cereus* to antibiotics before and after freeze storage. The zone of inhibition around each antibiotic disk was measured in millimeters.

B. cereus						
Antibiotic	Baseline	Week 1	Week 2	Week 3	Week 4	Week 5
Augmentin	9 mm	9 mm	11 mm	10 mm	9 mm	11 mm
Bacitracin	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm
Chloramphenicol	23 mm	25 mm	23 mm	23 mm	23 mm	24 mm
Nalidixic Acid	15 mm	14 mm	17 mm	15 mm	15 mm	17 mm
Novobiocin	17 mm	13 mm	15 mm	15 mm	18 mm	18 mm
Optochin	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm
Penicillin	0 mm	7 mm	6 mm	0 mm	0 mm	9 mm
Polymyxin	8 mm	9 mm	8 mm	8 mm	8 mm	8 mm
Tetracycline	14 mm	15 mm	17 mm	15 mm	15 mm	15 mm
Vancomycin	15 mm	15 mm	16 mm	15 mm	19 mm	15 mm

E. coli was susceptible to Tetracycline, Chloramphenicol, Nalidixic acid, Augmentin, and Polymyxin and resistant to Penicillin, Bacitracin, Vancomycin, Novobiocin, and Optochin (Table 3).

Table 3: Susceptibility of *Escherichia coli* to antibiotics before and after freeze storage. The zone of inhibition around each antibiotic disk was measured in millimeters.

E. coli						
Antibiotic	Baseline	Week 1	Week 2	Week 3	Week 4	Week 5
Augmentin	19 mm	19 mm	18 mm	18 mm	19 mm	18 mm
Bacitracin	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm
Chloramphenicol	21 mm	22 mm	20 mm	22 mm	22 mm	20 mm
Nalidixic Acid	20 mm	21 mm	18 mm	18 mm	19 mm	19 mm
Novobiocin	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm
Optochin	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm
Penicillin	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm
Polymyxin	13 mm	13 mm	12 mm	12 mm	13 mm	12 mm
Tetracycline	20 mm	21 mm	20 mm	20 mm	20 mm	20 mm
Vancomycin	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm

S. aureus was susceptible to Chloramphenicol, Vancomycin, Tetracycline, Nalidixic acid, Novobiocin, Penicillin, Polymyxin, Augmentin, and resistant to Bacitracin, and Optochin (Table 4). There was no significant change in antibiotic resistance in any of the species examined over the 5 week experimental period.

Table 4: Susceptibility of *Staphylococcus aureus* to antibiotics before and after freeze storage. The zone of inhibition around each antibiotic disk was measured in millimeters.

S. aureus						
Antibiotic	Baseline	Week 1	Week 2	Week 3	Week 4	Week 5
Augmentin	39 mm	40 mm	39 mm	40 mm	38 mm	38 mm
Bacitracin	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm
Chloramphenicol	20 mm	25 mm	23 mm	20 mm	19 mm	20 mm
Nalidixic Acid	13 mm	12 mm	13 mm	13 mm	12 mm	12 mm
Novobiocin	27 mm	25 mm	25 mm	26 mm	21 mm	26 mm
Optochin	0 mm	0 mm	0 mm	0 mm	0 mm	0 mm
Penicillin	38 mm	39 mm	43 mm	45 mm	37 mm	40 mm
Polymyxin	8 mm	7 mm	8 mm	8 mm	8 mm	8 mm
Tetracycline	25 mm	26 mm	26 mm	26 mm	23 mm	27 mm
Vancomycin	19 mm	19 mm	18 mm	16 mm	11 mm	20 mm

Confirmation of identification of *S. aureus* after week 5

With a positive urease result for *S. aureus* during week five, it was necessary to confirm the identification of *S. aureus* in the urea agar. Inoculation of SBA with the bacteria from the positive urease test yielded a clear area around the colonies, indicating a β–hemolytic bacterium. Two colonies, one from week one and the other from week five, were Gram stained to reveal the bacteria were Gram positive Staphylococci. *S. aureus* from week one and week five inoculated onto MSA grew and were able to use mannitol in acid fermentation. However, inoculation of MacConkey agar yielded no growth, ruling out contamination by Gram negative enteric bacteria (which are frequently urease positive). These results confirmed the identity of the bacteria from week five as *S. aureus*. The urea agar analysis was repeated using bacteria from the Kirby-Bauer plates from week one through five that had been stored at 4 °C. The results of these inoculations indicated a gradual induction of urease activity correlating to the length of time in freeze storage (figure 1).

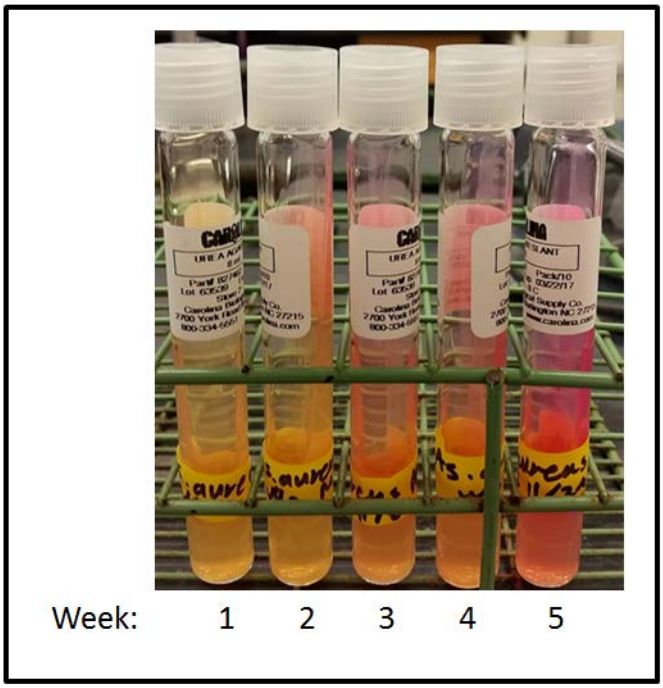

Figure 1. **Induction of urease activity in *S. aureus* following freeze storage**. Although the strain of *S. aureus* used in this study was initially urease-negative, inoculation of urea agar was decidedly urease positive after week five of freeze storage. New urea agar was inoculated with *S. aureus* from each week of the experiment. Results indicate long term storage of these cells leads to an induction of urease in *S. aureus*.

Conclusions

This study investigated the stability of a set of biochemical characteristics after freeze-thaw storage, using a group of standard tests on three bacteria species*: B. cereus, E. coli*, and *S. aureus*. Although freezing is a very common, inexpensive way to store bacterial stocks long term, there is the possibility that the population of recovered cells differs from the parent population due to selection pressure imparted by the freeze-thaw cycle. However, over the experimental period of 5 weeks, no significant changes were found for most of the physiological characteristics. While there was no significant variance in antibiotic resistance with *B. cereus* or *E. coli, S. aureus* appeared to become more resistant to Vancomycin by week four, as indicated by a much smaller zone of inhibition (ZOI). However, results from week 5 with *S. aureus* did not significantly differ from the overall mean ZOI for this species and antibiotic combination. Without further replication or investigation, it is not possible to determine if the week 4 result is significant, or due to degraded antibiotic in that specific disk.

It appeared that freeze storage altered the ability of *S. aureus* to metabolize urea, as shown by the positive urea agar assay on week 5. However, a literature review revealed that the *S. aureus* genome includes a urease gene that can be induced by stressors such as ethanol or acid (Bore et al., 2007; Korem et al., 2010). It appears long term freeze storage induces urease gene expression in *S. aureus* in much the same way it is induced when exposed to ethanol or acid.

The results presented above support the hypothesis that the basic bacterial characteristics will not change after freeze-thaw treatment. However, more experimentation

might reveal that freeze storage may in fact select for new characteristics in other species of bacteria. When conducting these experiments, investigators must consider the possibility of the induction of previously unexpressed traits. It was challenging to find additional previous papers addressing the same concern. Also, other chemicals can be used in cryopreservation, and such chemicals may affect bacterial populations differently than glycerol following freezing. This experiment was only five weeks in duration, and examined only glycerol, thus more extensive, long-term studies (months to years) may yield different results.

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Bacterial contamination on paper towel dispenser levers is positively correlated to traffic in college restrooms

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Abstract

Bacterial abundance on paper towel dispenser levers is pertinent to public health as these levers are a potential source of infection. Despite this, there remains a dearth of studies on bacterial presence on the levers of paper towel dispensers. This study reveals an abundance of bacteria on the levers of paper towel dispensers in bathrooms on a college campus in Raleigh, North Carolina. Roughly 85% of samples from high-traffic areas produced colonies that were too numerous to count when grown on convex Replicate Organism Detection and Counting plates with tryptone soy agar. Two main solutions are proposed to decrease bacterial presence on paper towel dispenser levers and avoid potential adverse health outcomes. The first is to replace lever-based dispensers with hands-free or auto-feed paper towel dispensing systems. An alternative solution of scheduled and frequent cleaning of the levers with antibacterial solution is more economically feasible, especially in areas of organizations that cannot afford hands-free or auto-feed paper towel dispensing systems. Further investigation is needed to assess the usefulness of cleaning paper towel dispenser levers as a means of reducing the risk of bacterial contamination for restroom patrons.

Introduction

Proper hand-washing and general hygienic techniques are widely considered to be the most effective means of eliminating the spread of diseases (Allegranzi and Pittet, 2009; Larson, 1988; Michaels et al., 2001). The opportunity for bacterial contamination during and after hand-washing has been studied previously, and has revealed that many bathroom surfaces may be particularly dangerous (Griffith et al., 2003; Harrison et al., 2003a). Surfaces of particular interest in the spread of potentially hazardous microbes are those handled following the rinsing

of hands, including the sink faucet handle, inside the bathroom door handle, and the paper towel dispenser levers. All of these surfaces have the potential to contain numerous bacteria. However, touching the sink faucet handle and bathroom door handle can be avoided by using a paper towel as a physical barrier. Unfortunately, touching the paper towel dispenser lever cannot be avoided in this same way, and this lever could therefore serve as a vehicle for pathogen transfer. Due to the public health implications, the microbial abundance on bathroom paper towel dispenser levers at a college campus in Raleigh, North Carolina was examined. The hypotheses were as follows:

Firstly, it was predicted that if three areas of the levers (left, center, and right) were sampled, then all three areas would produce approximately the same number of plates which grew colonies too numerous to count (TNTC). Secondly, the ratio of bacterial abundance from male bathrooms to female bathrooms would be roughly proportional to the ratio of male to female students on the campus, as a reflection of proportional traffic occurring in restrooms. Thirdly, due to the dynamic nature of the restrooms, it was predicted that at least half of the samples taken from high-traffic areas would have bacterial abundance that was TNTC.

Materials and Methods

There is always a risk for the unintended release of and/or exposure to microbes when cultivating bacteria. Proper and standard laboratory safety precautions (e.g. proper personal protective gear, removal of jewelry while performing lab work, minimizing distractions while working with samples) were therefore followed to minimize this risk. Before beginning the experiment, approval was obtained from the appropriate administrators for sampling

restrooms for bacterial abundance. Administration also provided data regarding male to female student ratio on the campus sampled.

For a bacterial growth medium, small convex agar plates were used. The agar plates were Replicate Organism Detection and Counting plates (RODAC) with tryptone soy agar (TSA) including Tween 80 and lecithin. Three plates were used for each paper towel dispenser sampled: one for the left portion of the lever, another for the middle portion, and a third for the right portion of the lever (see Figure 1). Each agar plate was pressed onto a third of the lever, and labeled accordingly. Additionally, labeling distinguished whether the paper towel dispenser was "near" the door, or "far" away from the door, as some bathrooms had two paper towel dispensers. Lastly, the building name, floor number, and bathroom gender were noted on each plate. After sampling, the stacks of plates were all simultaneously placed inside a 37°C

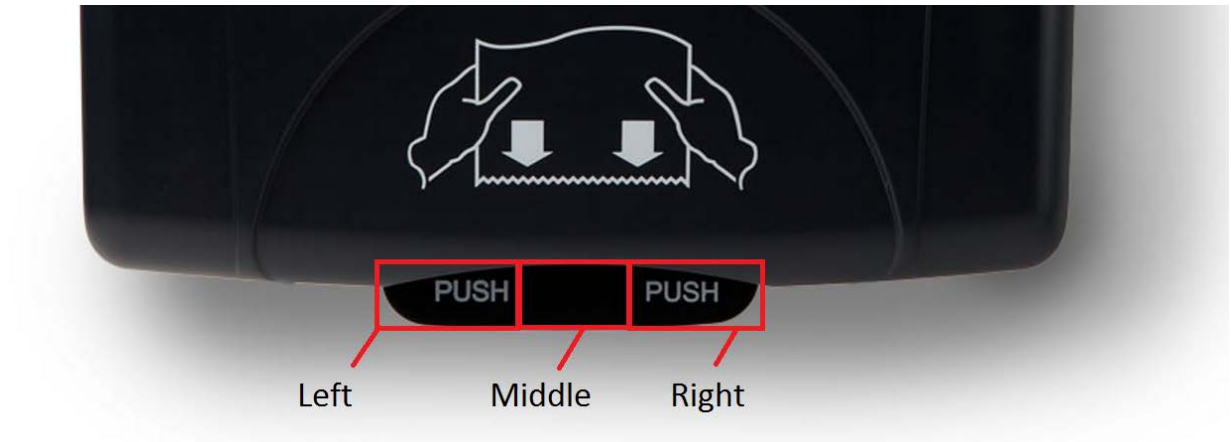

Figure 1: Image of the type of paper towel dispenser sampled for this project. Three different areas were sampled from each paper towel dispenser lever, left of center, center and right of center.

incubator. All the plates were simultaneously taken out of the incubator after 72 hours of incubation and the number of colonies on each plate was counted and recorded alongside the identifying characteristics of the plate (building name, floor number, male or female bathroom, near or far dispenser, and left/middle/right section of lever).

Results

Analysis of the plates used to sample the left, middle, and right portions of the paper towel dispenser levers revealed that these three areas of the levers produced approximately the same number of TNTC plates (Table 1). There were 14 TNTC left sections, 17 TNTC middle sections, and 18 TNTC right sections, out of a total of 32 plates from each section. In looking at these areas while also considering if they were on the near or far dispenser, the greatest difference was in the frequency of TNTC in the "far right" and "near right" areas, which were 4 and 11, respectively.

Table 1. TNTC Plates on Near and Far Dispensers

Side and section	Number of TNTC plates
Far left	6
Far middle	8
Far right	4
Near left	6
Near middle	7
Near right	11

This table displays the number of TNTC plates obtained from the left, middle, and right sections of the levers on paper towel dispensers located "near" or "far" from the door to the restroom. This data supports the first hypothesis, asserting that the left, middle, and right sections of the levers produced approximately the same number of TNTC plates.

The gender ratio on the campus sampled was 511 males to 2166 females at the time of sampling. The number of samples taken from male and female bathrooms was not the same, because some female bathrooms contained two paper towel dispensers while the companion male bathrooms contained a single paper towel dispenser. Due to this disparity, the percentage of sample plates which were determined to be TNTC was compared instead. Out of 51 female bathroom samples, 29 of the samples were TNTC (56.86%). Out of 45 male bathroom samples, 20 of the samples were TNTC (44.44%). The ratio of TNTC samples from male to female bathrooms (1:1.28) was not proportional to the ratio of male to female students on the campus sampled (1:4.24).

Data from bathrooms located in high-traffic areas was considered. The first two areas of interest considered were floors 1 and 3 of the Health Science Building (HSB), because these floors contain entrances and exits to the building. A majority (21 out of 24) of the agar plates used to sample the bathrooms on these floors resulted in growth of colonies that were TNTC (Table 2). The first floor of the HSB yielded 11 plates out of 12 (91.67%) that were TNTC, while the third floor of HSB produced 10 plates out of 12 (83.33%) that were TNTC. Other areas of interest included bathrooms in the Health Education Building (HEB), which are located nearest to the college library and are also accessed by the students from the high school attached to this building. These areas produced 6 plates out of 6 (100%) that were TNTC. The last area with considerable bacterial abundance was the second floor of the Allied Health Building (AHB). This floor houses the offices of many faculty members, and is therefore frequently visited by both professors and students. The bathrooms on the second floor of AHB produced 6 plates

out of 9 (66.67%) that were TNTC. These results support the third hypothesis, because 85.71% of the plates used to sample high-traffic area produced colonies that were TNTC.

These results were compared to the results from low-traffic bathrooms, or those that had no obvious major attractions nearby. Floors 2, 4, and 5 of HSB produced only 13 plates out of 36 that were TNTC (36.11%), and floors 1 and 3 of AHB produced only 3 plates out of 21 that were TNTC (14.29%).

Table 2. Percentages of TNTC Plates on Paper Towel Dispenser Levers.

Floor 5			33%
Floor 4			42%
Floor 3	0%		**83%**
Floor 2	**67%**		33%
Floor 1	25%	**100%**	**92%**
Building name	*AHB*	*HEB*	*HSB*

Key
X>=67%
33%<X<67%
X<=33%
X = percent of TNTC plates

This table represents the percentages of TNTC plates resulting from the paper towel dispenser levers on each floor in each building on a health science campus in Raleigh, NC. Allied Health Building (AHB) floor 2, Health Education Building (HEB) floor 1, and Health Science Building 1 (HSB) floors 3 and 1 were all considered high-traffic areas due to the presence of entrances or attractions (i.e. library in HEB floor 1 and professor office space on AHB floor 2). This table shows the direct, positive correlation between bacterial abundance and traffic level in public restrooms, specifically bacterial abundance on paper towel dispenser levers.

Overall, 96 total samples were taken from 32 bathrooms. Following incubation for 72 hours on non-selective agar, 49 plates were TNTC (51.04%), 1 plate contained 51 colonies (1.04%), 27 plates contained between 10 and 50 colonies (28.13%), and 19 plates contained under 10 colonies (19.79%). Thus a total of 77 plates contained greater than 10 colonies (80.21%). Many factors can influence both the rate of growth and overall quantity of bacteria

on a plate at any time point. Even given this variability in growth, our results showed the presence of high numbers of bacteria (both in TNTC percentages and countable colony averages) in the locations sampled, especially those with high traffic.

Conclusions

The three areas of the paper towel dispenser levers (left, middle, and right) contained roughly the same bacterial abundance, as indicated by the roughly equivalent number of TNTC plates grown from samples in these areas. This suggests that hand dominance (i.e. right versus left handedness) does not have a significant impact on the bacterial abundance on levers, though further research into hand-to-lever technique would be necessary to make a clear conclusion on the true impact of hand dominance on lever contamination.

There were more TNTC plates from female bathrooms than male bathrooms, but there were more female than male students on the campus at the time of sampling. In this way, the number of TNTC plates from female to male bathrooms trended with the ratio of female to male students on the campus sampled. However, the ratios were not proportional to one another. The female to male student ratio was 4.24:1, while the female to male TNTC plate ratio was 1.28 to 1. This trend may be causal in nature, meaning that the heavier traffic in female versus male bathrooms caused the female to male TNTC plate ratio to be skewed, or this trend may instead be a result of other unknown factors. The original hypothesis was that the ratio of bacterial abundance from female to male bathrooms would be roughly 4.24:1, and though both do trend in the same direction, the ratio of TNTC plates is not so stark. It is worth noting that there still could be four times as many colonies on female plates, but this could

have gone undetected because TNTC was the assigned method of comparing bacterial abundance in this experience.

It is reasonable to postulate that bathroom traffic would depend on the location of each bathroom, and that there would be high bacterial abundance in areas of high traffic. Roughly 85% of the samples taken from high-traffic areas produced colonies that were TNTC, as opposed to roughly 28% in low-traffic areas, which suggests a direct, positive correlation between traffic level and bacterial abundance in bathrooms on campus.

This experiment points to paper towel dispenser levers as potential vehicles for bacterial contamination, and should be cause for concern for public health officials and organization administrators. There are two main solutions to minimize or eliminate the risk for infection created by the presence of bacteria on paper towel dispenser levers.

The first solution is to replace the current lever-based paper towel dispensers with either motion sensor-based (hands free) paper towel dispensers or auto-feed paper towel dispensers. This strategy was implemented in the construction of the newest building on the campus sampled. Theoretically, the auto-feed paper towel dispensers provide patrons a less-risky bathroom experience; however, there is evidence that auto-feed paper towel dispensers still pose a bacterial risk to patrons, due to the tendency of paper towels to dispense improperly. One study found that certain brands of folded paper towels malfunction less often than other brands, and that malfunctions of jamming, towels falling to floor, and incidental contact with paper exits increases the risk of bacterial exposure for patrons (Harrison et al., 2003b). A way to reduce the cost of this first option is to only replace the paper towel dispensers in high-traffic areas and to stock them properly. Harrison's study also examined the

economic impact of different brands of folded paper towels, and found that one brand of paper towels was almost 30 times more wasteful than another brand (Harrison et al., 2003b). The second option is to have the janitorial staff clean the paper towel dispenser levers with standard antibacterial solution during regular bathroom cleanings. A similar yet even more economical option is to have the janitorial staff clean only the paper towel dispenser levers in high-traffic bathrooms. The option of cleaning the levers is more economically viable than fully replacing the dispensers because it eliminates the need to invest significant capital, requires negligible man-hours to execute, and demands the use of only a small amount of solution. Unfortunately, dispensers will be used and re-contaminated throughout the day by multiple patrons; therefore, this method will not eliminate the risk entirely.

Further investigations which could help administrators choose between these options might examine the effectiveness of cleaning the paper towel dispenser levers with standard antimicrobial solution during regular bathroom cleanings. If such an experiment suggests that periodic cleaning of the levers is sufficient to significantly reduce bacterial presence throughout the day thereby significantly lowering risk to bathroom patrons, then the option of cleaning the levers would be a clear choice to improve upon public health on campus.

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