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Distribution of Antimicrobial Resistance Indicators in Wastewater and Demographic Risk Factors Across Diverse Atlanta Metropolitan Communities

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Bachelor of Science
University of California, San Diego
2021

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An abstract of
A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Public Health
in the Department of Epidemiology
2023

Abstract

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By Caroline Homa Sheikhzadeh

Background Antimicrobial-resistant (AMR) bacteria are responsible for 2.8 million infections and 35,900 deaths annually in the U.S. alone, with alarming growth rates of global AMR prevalence. The relationship between social determinants of health and AMR prevalence is still understudied, and there is a critical need to establish standards for AMR quantification and risk factor surveillance. This paper aims to explore the use of wastewater surveillance to assess relationships between increased AMR prevalence and hypothesized risk factors across diverse communities in Atlanta.

Methods In December 2022, wastewater samples were collected from seven wastewater treatment plants (WWTPs) in metropolitan Atlanta. Samples were cultured for third-generation-cephalosporins-resistant (3GC) and carbapenem-resistant (CR) *E. coli* and *Klebsiella pneumoniae*, and fluoroquinolone (FQ)-resistant Enterobacterales. The U.S. Census Bureau's 2020 American Community Survey was used to explore age, race, sex, median household income, poverty, and uninsured variables as they relate to AMR prevalence in communities captured by WWTPs. Outcomes were analyzed using simple and multiple linear regression (SLR, MLR) models. The reduced MLR model included female, foreign-born, uninsured, and age under 5 as covariates.

Results In the MLR, we found a significant relationship between FQ-resistant Enterbacterales and foreign-born status (0.21-CFU/100mL increase with 1% increases in foreign-born status). The SLR models found significant relationships between both CR *E. coli* and FQ-resistant Enterobacterales and population over 75 (52 and 2.4-CFU/100mL decrease, respectively, with 1% increases in population 75 and older). There was also a significant relationship between CR *E. coli* and Non-Hispanic (NH) Asians (5.8-CFU/100mL increase in CR *E. coli* with 1% increases in NH Asians).

Conclusion Increases in concentrations of AMR bacteria cultured from wastewater were related to increases in percentages of foreign-born and NH Asians and decreases in percentage 75 and older. While these are the only significant relationships, further analysis with larger sample sizes could provide more insight into potential relationships between AMR prevalence and other risk factors. These results support the need for standardized methods of AMR surveillance and to better understand risk assessment frameworks for targeted resource allocation in hopes to slow global AMR prevalence rates.

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1 INTRODUCTION

Global burden of bacterial infections & antibiotic resistance in bacteria

Bacterial infections are a major cause of global morbidity and mortality as the second leading cause of death globally (Vos et al., 2020). In 2019, a study by the Global Research on Antimicrobial Resistance (GRAM) Project estimated that there were 7.7 million global deaths associated with 33 common bacterial infections, accounting for one in eight global deaths (Murray et al., 2022). Among these deaths, more than 1.27 million were attributed to antimicrobial-resistant bacterial infections. The role of antimicrobial resistance (AMR) in the global burden of disease is projected to continue growing; the United Nations (UN) estimates that by 2050 up to 10 million annual global deaths could be attributed to AMR (Environment, 2023). National estimates published by the Centers for Disease Control (CDC) indicate that each year, at least 2,868,700 infections and 35,900 deaths are caused by antibiotic-resistant (AR) bacteria and fungi in the United States (CDC, 2022).

Prevalent strains of antimicrobial-resistant bacteria and genes

As of 2019, the six most prevalent pathogens responsible for AMR-related mortality are *Escherichia coli (E. coli),* followed by *Staphylococcus aureus*, *Klebsiella pneumoniae (K. pneumoniae)*, *Streptococcus pneumoniae*, *Acinetobacter baumannii (A. baumannii)*, and *Pseudomonas aeruginosa*. In 2019 alone, these pathogens were responsible for 930,000 deaths attributable to AMR and 3.6 million deaths associated with AMR. An additional six pathogens caused 50,000-100,000 deaths each: drug-resistant tuberculosis, third-generation cephalosporin (3GC) resistant *E. coli*, carbapenem-resistant (CR) *A. baumannii*, fluoroquinolone (FQ) resistant *E. coli*, CR *K. pneumoniae*, and 3GC-resistant *K. pneumoniae* (Murray et al., 2022).

Development of antimicrobial agents

Bacterial microorganisms have been a concern for human health for centuries. The first antibiotic agent effective against bacterial pathogens was penicillin, developed in the 1940s (Abushaheen et al., 2020). Since then, there have been several classes of antibiotics developed to fight bacterial infections. Most can be classified into six groups: penicillins, cephalosporins, aminoglycosides, tetracyclines, macrolides, and fluoroquinolones. These antimicrobial agents have proved effective in fighting a variety of pathogenic microorganisms. Each type of antibiotic has specific uses and sometimes side effects (*Antibiotics*, 2017). Broad-spectrum antibiotics (e.g., amoxicillin) are more commonly used for treatment of bacterial infections and have minimal side effects, while last-line antibiotics (e.g., carbapenems) are only used in severe cases and have serious side effects.

Mechanisms from antibiotic use to increasing AMR prevalence

Widespread overuse and misuse of antimicrobial agents have caused an increase in antimicrobial-resistant bacteria (Wushouer et al., 2018). Clinical diagnostics for bacterial infections have yet to match the growing need for rapid antibiotic sensitivity analysis completed before treatment administration. Current testing methods are time-costly and force clinicians to administer uninformed treatment of broad-spectrum antibiotics, which can prove to be ineffective if the pathogen is resistant to the given antibiotic(s). Without punctual diagnostic results regarding the sensitivity of the target pathogen, the cycle of improper use of antimicrobial agents continues. AMR characteristics in bacteria allow organisms to survive and remain vital regardless of exposure to antimicrobial agents. The developed resistance prevents the use of antibiotics as effective treatments against the spread and progression of bacterial infections.

When a bacteria is resistant to a specific antibiotic, the infected host cannot be treated with that antibiotic, narrowing the available treatment options. The more AMR genes that are seen in a clinically-relevant bacterium, the less antibiotics that are effective, and the more difficult it will be to treat. These "superbugs" can develop after repeated exposure to antibiotics, which support the natural selection of bacteria with mutations that are not sensitive to antimicrobial exposure and therapeutics. Temporal trends of AMR bacteria have shown increases in prevalence of resistance to both common antibiotics (e.g., amoxicillin-resistant *E. coli*, 3GC-resistant *E. coli*), as well as last-line antibiotics (e.g., FQ-resistant *Salmonella* spp. and CR *Pseudomonas aeruginosa*) (Mhondoro et al., 2019). As these AMR genes continue to circulate in bacterial populations globally, there is an alarming complexity developing in AMR pathogens and treatments required to overcome them (Environment, 2020).

Factors affecting the rise in AMR bacteria

There are several literature-identified factors contributing to the increase in AMR prevalence, and complexity of multi-resistance. These include the genetic evolution of bacteria, misuse and overuse of antimicrobial agents, agricultural use of antibiotics and subsequent environmental contamination, increases in income levels, and global connectivity (Dadgostar, 2019).

Biological factors - The spontaneous mutation of bacteria and horizontal gene transfer capabilities between bacteria have contributed significantly to the widespread development of bacteria that contain AMR genes and are no longer sensitive to antimicrobial agents. Improper use of antibiotics, including overuse and inaccurate dosages, also supports the development of bacterial antimicrobial resistant genes. This can render treatments to be ineffective and cause

common bacterial infections to become life-threatening, increasing morbidity and mortality rates (Kaprou et al., 2021).

Global connectivity - The interconnected nature of modern society with more efficient traveling routes for humans, trade, and animals, has supported the ease of spread of AMR world-wide. Human-specific travel between isolated areas of the globe can drive the spread of antimicrobial resistance as humans become exposed to and colonized by resistant pathogens. These bacteria can then spread to an entirely new population around the world with the efficiency of present-day travel capabilities (Castro-Sánchez et al., 2016). While there are some regulations on antibiotic access in the United States and other countries, worldwide regulations are variable and areas of the world with less restrictions on antibiotic use can lead to public overuse of antibiotics. This increases the burden of AMR in those areas, as well as areas that may have antibiotic restrictions, but have civilians who travel to less restrictive areas where they may purchase/use non-prescription antibiotics without clinical consultation (Jani et al., 2021). Variability in antibiotic consumption, access to clean water and sanitation resources, and access to healthcare resources globally can also impact the prevalence of AMR in local communities (Frost et al., 2019). The combination of global connectivity and a lack of global uniformity in antibiotic stewardship contribute to increasing rates of AMR prevalence.

Antimicrobial presence in the environment - Antimicrobial agents are commonly used in the agriculture industry (e.g., livestock and crop production) for pathogen treatments and in food and water sources for animals (Environment, 2023). The established relationship between antibiotic usage and increases in AMR microorganisms highlights the public health problem caused by

environmental contamination of antibiotics. Environmental pollution with antimicrobials in areas where water and wastewater treatments are inadequate raises concerns about close human contact with polluted waters. This could result in a consumption-excretion cycle, in which poor control of antimicrobial pollutants or inadequately treated wastewater is used in the irrigation of farmland or for crop fertilization. Addressing cycles of excessive spread of antimicrobials in the environment should be a priority in both surveillance and intervention efforts given their participating role in global AMR prevalence (Environment, 2023).

Rising income - Increases in income levels and subsequent increased animal protein consumption in developing countries are also associated with rises in agricultural antimicrobial agent usage. Rises in Gross Domestic Product (GDP) and low and middle-income countries' living standards have been associated with an overconsumption of antibiotics (Dadgostar, 2019). These trends are particularly prevalent in combination with a lack of effective antibiotic stewardship programs, and lower antibiotic use education.

Antibiotic use and demographic factors

Previous studies have examined the distribution of antibiotic prescription rates among global communities, to identify risk factors for increased antibiotic use. Prescription and prescription fill rates for antibiotics are often used as a proxy for antibiotic use, with studies analyzing demographic characteristics in populations with increased antibiotic prescription rates compared to those with lower rates. Recent studies have shown that prescription rates for women are higher than that for men and women are more likely than men to receive an antibiotic prescription in their lifetime (Schröder et al., 2016). Other studies looking at the age distribution of prescriptions

per 1,000 persons, found that those who are 75 and older receive more prescriptions compared to those in the population aged 65 to 74 years old (Kabbani et al., 2018). These studies have indicated a correlation between gender and age demographic factors and differences in antibiotic use rates in a population. The correlation between increased antibiotic use and AMR prevalence in the context of these SDOH studies, suggest that further analysis of these demographic factors in relation to AMR outcomes is warranted. However, the use of antibiotic prescriptions as a proxy for antibiotic use or AMR prevalence has limitations, as it fails to account for non-prescription and misuse of antibiotics.

Treatment options for AMR pathogens

Treatment of AMR bacterial infections is complex given the inability of common antibiotics to work against this grade of infections. Specific AR pathogens have been targeted by international research campaigns scrambling to find new drugs that can be used to treat increasingly AMR bacteria (Butler et al., 2022). Ongoing research on nontraditional antibacterial treatments includes the use of antibodies, bacteriophages, phage-derived enzymes, microbiome-modulating agents, and immunomodulating agents (Butler et al., 2022). There have been efforts to develop vaccines that increase the sensitivity of AMR pathogens to antibiotics. They have been seen to be successful with *Streptococcus pneumoniae* and *Haemophilus influenzae*, however, this area of research needs further development for a larger range of pathogens (Alghamdi, 2021). However, research into innovative solutions to meet treatment needs of AMR patients is both financially and temporally costly. As an example, the un-tapped exploration of global tropical forests may help in the discovery of plant resources that provide solutions to fighting AMR pathogens, but this research requires significant financial investment to investigate (Yoshikawa, 2002).

The financial burden of AMR

In addition to clinical implications, diagnosis and treatment of AMR bacterial infections is significantly more expensive than diagnosis and treatment of infections without resistance, increasing the financial burden on healthcare systems (Alanis, 2005). The UN predicted that by 2050, AMR infections could be responsible for not only an increasing number of global deaths but could use $3.4 trillion USD of the GDP annually and cause 24 million people to drop below the national poverty line into extreme poverty over the next decade (Environment, 2020). This increase in financial hardship connected to AMR is due in part to high healthcare costs from increased hospital admissions, morbidity, and complex drug usage (Dadgostar, 2019).

Existing methods of AMR surveillance

Many surveillance systems exist for AMR pathogens, and each surveillance method provides different information regarding AMR characteristics. Clinical and food sectors working to monitor AMR have historically gathered case-specific information on individual AMR pathology (Pruden et al., 2021). AMRFinderPlus, developed by the National Center for Biotechnology Information, is one identification tool for AMR pathogen detection (e.g., AMR genes, resistance-associated point mutations) (*AMRFinderPlus - Pathogen Detection - NCBI*, n.d.). The NCBI pathogen detection pipeline works to identify key AMR components of pathogen genomes from various ongoing surveillance and research projects. Sources for AMRFinderPlus include food, environmental, and patient samples, including those for foodborne, hospital-acquired, and other clinically infections pathogens. The National Antimicrobial Resistance Monitoring System (NARMS) is another surveillance system for AMR. NARMS is a tool that primarily assesses the changes is AMR transmitted through food, water, animals, person-to-person contact, and

environmental contamination (*Antibiotic Resistance and NARMS Surveillance | NARMS | CDC*, 2022). This system supports the understanding of human health implications arising from AMR bacteria present in the food production industry in the United States (Karp et al., 2017).

Wastewater surveillance of AMR

Environmental surveillance of AMR has recently gained momentum, in part due to infrastructure development for monitoring SARS-CoV-2 virus prevalence in wastewater (Pruden et al., 2021). Wastewater monitoring provides aggregated data that represents the population contributing to a sewer system, encompassing not only clinical cases, but also capturing asymptomatic cases, those not tested for AMR bacteria, or those who did not seek or have access to healthcare (Farkas et al., 2020). The scale of AMR surveillance informs researchers of the prevalence of AMR targets without the necessity for individual clinical testing, saving costs with higher potential for accuracy.

A systematic review of wastewater AMR surveillance systems on human communities found that among the various quantification methods for AMR prevalence in the environment, studies with time and location matching between wastewater sampling and human population data, as well as using composite sampling of influent lines and sampling longitudinally (> 12 months) showed the highest agreement between wastewater and human compartment (e.g., clinical sampling) surveillance compared to systems that did not have these components (Chau et al., 2022).

AMR quantification methods

There are several methods currently used for processing samples to quantify the presence and concentration of targets associated with AMR in wastewater. AMR monitoring in water

environments commonly utilizes culture-based, qPCR-based, or metagenomics-based methods (Liguori et al., 2022). To date, studies measuring human AMR prevalence in wastewater most commonly utilize phenotypic methods which quantify observable characteristics including drug susceptibility (Chau et al., 2022), genotypic methods which measure genes or mutations that are known to decrease drug susceptibility, or both detection methods (*Clinical Use of Genotypic and Phenotypic Drug Resistance Testing to Monitor Antiretroviral Chemotherapy | Clinical Infectious Diseases | Oxford Academic*, n.d.). Phenotypic methods include both manual protocols with agar dilution, gradient tests, disk diffusion, and broth microdilution and automated resistance detection platforms such as VITEK 2 COMPACT, Sensititre, and ARIS 2X (Kaprou et al., 2021). Molecular-based protocols for AMR detection include PCR-based, isothermal amplification, and DNA microarray methods. Other less conventional methods that can be used include genome sequencing and metagenomics, MALDI-TOF mass spectroscopy, and microfluidic technology. Digital droplet PCR (ddPCR), is a method of PCR used for direct quantification that also has the benefit of high sensitivity, however, there is still limited published work on the use of ddPCR for AMR detection.

Gaps in the Literature

Wastewater surveillance of AMR genes has shown promise in recent studies to satisfy the limitations of clinical data, however, there are still many gaps in the comparison of the methodology of AMR gene quantification (Majeed et al., 2021). Inconsistency in reported study features, limited sample sizes, and significant heterogeneity in the approaches used have limited the conclusions regarding AMR transmission and prevalence. Standards of best practice in both culture- and molecular-based AMR surveillance are not well established, and prevent the

development of cohesive, centralized AMR data integration across systems that could benefit from combination of resources and datasets (Pruden et al., 2021).

Rising concerns for AMR prevalence has raised interest in risk factors for AMR prevalence. The known positive relationship between antibiotic use and AMR prevalence (Dadgostar, 2019) has been used in several studies to assess antimicrobial resistance, using antibiotic prescription rates as a proxy measure for antibiotic use. This remains a limitation to the accuracy of data connecting AMR prevalence to risk factors, due to inaccuracies between prescription writing and true antimicrobial use, as well as a limitation on reported patient AMR cases (Olesen & Grad, 2018). Therefore, the true social determinants of health (SDOH) as they could relate to AMR prevalence remains severely understudied, and further development of environmental surveillance systems could have the potential to fill this gap. Thus, in this study we explore the relationship between AMR and demographic factors using wastewater measurements to provide information on the likely distribution of AMR across Atlanta.

Addressing AMR Prevalence and SDOH in Atlanta, Georgia

In 2017, the World Health Organization (WHO) published grouped rankings of the most and least critical groups of priority AMR pathogens. Surveillance targets for AMR genes can be informed by the critical priority pathogen groups identified by WHO. The critical priority group of pathogens includes carbapenem-resistant *Acinetobacter baumannii*, carbapenem-resistant *Pseudomonas aeruginosa*, carbapenem-resistant and 3GC-resistant *Enterobacteriaceae* (*WHO Global Priority Pathogens List of Antibiotic-Resistant Bacteria - Combat AMR*, 2021).

According to insight provided by Gillian Smith and Dr. Jesse Jacob, CDC convenience sampling of AMR in Atlanta through 2019 showed *Klebsiella pneumoniae carbapenemase* (KPC) as the most common carbapenem followed by New Delhi Metallo-β-Lactamase (NDM), and noting several clinical cases of OXA-48-producing carbapenemase (Personal communication via email, 20 June 2022). This information provided on the prevalent bacterial strains in Atlanta in addition to global priorities for AMR pathogens contributed to the choice of AMR gene and bacterial culture targets for this paper's data collection and analysis. Analysis of associations between these critical outcomes and community-specific risk factors is crucial to inform public health decisions and AMR infection prevention efforts. This paper aims to explore possible relationships between AMR prevalence and potential risk factors across diverse communities, in hopes of supporting future development of adaptable risk assessment frameworks using wastewater AMR surveillance (Pruden et al., 2021).

2 METHODS

Sample Collection

Wastewater samples were collected from seven wastewater treatment plants for this study, utilizing pre-existing wastewater sampling methods for wastewater surveillance programs in the metropolitan Atlanta area. These wastewater treatment plants (Figure 1) are distributed throughout Fulton County in the Atlanta metropolitan area. This analysis includes these seven wastewater treatment plant samples collected at a single time point (December 2022), each of which is a mutually exclusive community-level data point and avoids dependency or cross-over between samples.

Samples were available from three plants in the City of Atlanta managed by the Atlanta Department of Watershed Management (RM Clayton (RMC), South River (SOR), and Utoy Creek (UTC)). These samples were collected through Emory University's Center for Global Safe WASH COVID-19 wastewater monitoring program and are also affiliated with the WastewaterSCAN project, a national wastewater surveillance program. Samples were also provided from four additional plants by Fulton County Public Works in partnership with WastewaterSCAN (Big Creek (BIG), Camp Creek Basin (CMP), Johns Creek (JON)) (**Figure 1**). All samples were composite samples of influent wastewater entering the treatment plant. Samples were taken by operators and composited using an automated sampler over a period of 24 hours. Samples were then stored at 4℃ for 18-24 hours until each sample was processed with the two methods of quantification: bacterial culturing and PCR.

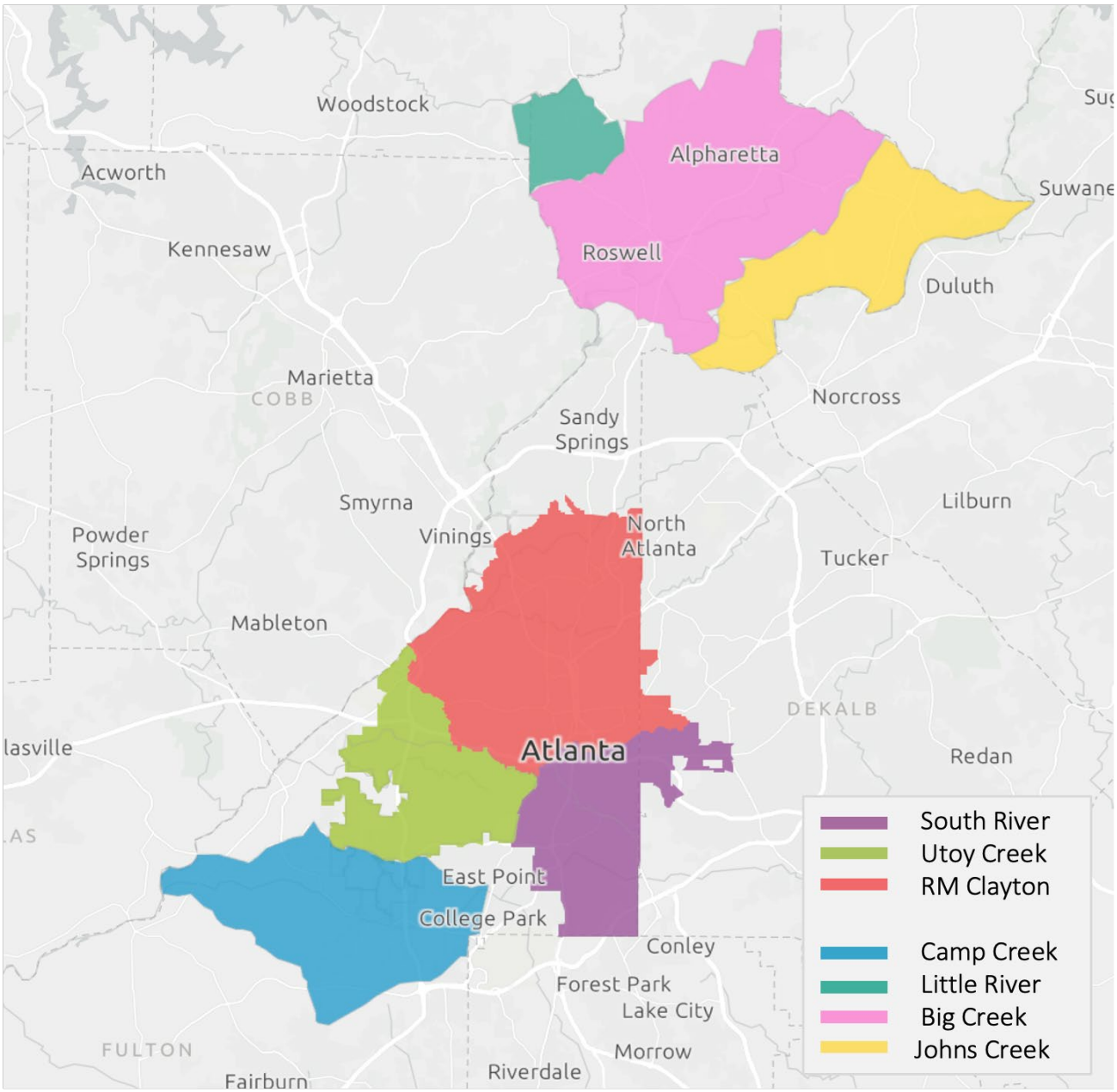

Figure 1. Map of Atlanta wastewater plant catchment areas (map base: Esri, HERE, Garmin, SafeGraph, METI/NASA, USGS, EPA, NPS, USDA | Esri, HERE, Garmin, SafeGraph, METI/NASA, USGS, EPA, NPS, USDA)

Genotypic Sample Processing

DNA Concentration & Extraction for PCR

Approximately 50 mL of each sample was centrifuged for 15 minutes at 4000 rpm. For PCR preparation, bacteria were concentrated from the supernatant of the sample using Nanotrap magnetic hydrogel nanoparticles (CERES NANOSciences, Virginia) and DNA was extracted from the supernatant of the sample using the MagMax Viral/Pathogen Nucleic Acid Isolation Kit (Thermo Fisher Scientific, Massachusetts). Both steps were performed on the KingFisher Apex platform (Thermo Fisher Scientific, Massachusetts). The parameters and steps of the concentration and extraction process were adopted from the Center for Global Safe WASH COVID-19 Detection Project (Sablon III et al., 2021). In the concentration step, Nanotrap magnetic particles bind to viruses and bacteria within a sample. This portion is then separated from the remaining supernatant using a magnet and then resuspended. The resulting lysate is then used in the extraction protocol, which includes a series of wash steps and the use of MagMax DNA/RNA binding beads to extract DNA and RNA from any viral and bacterial cells present in the sample. Following extraction, samples were stored at -20℃ for later analysis using PCR.

Gene Targets for qPCR Analysis Methods

Extracted DNA was processed using primers and probes from the *Streck AMR-D Kit, Beta Lactamase Kit* (Streck, Nebraska), targeting several antimicrobial resistance gene families: KPC, NDM, OXA-48, IMP, VIM, DHA, CMY-2, CTX-M-14 and CTX-M-15 (*ARM-D Kit, β-Lactamase*, n.d.). These assays were tested in 2021 along with assays from OpGen and Check-Points, to evaluate the ability of these assays to provide accurate identification of antimicrobial resistance loci in clinically significant Gram-negative bacteria. The results indicated that the Streck assay was robust for all gene targets included except for NDM (Brazelton de Cardenas et

al., 2021). While the *Streck AMR-D Kit* was developed for clinical samples, clinical assays are commonly adapted for use on environmental samples.

PCR Analysis Methods

There were three different multiplex Streck assays with three targets per assay. Each assay was combined with nuclease-free water and a SuperMix with buffer, dNTPs, MgCl2, and DNA polymerase into a master mix. For each reaction, 23 mL of this master mix was combined with 2 mL of extracted DNA to have a combined volume of 25 mL in each well. Each sample was run with duplicate wells, and a standard curve was run using dilutions of the Streck-provided positive controls. The mixtures placed in wells of a 96-well PCR plate were inserted into a C1000 Touch Thermal Cycler (BioRad, California) where specified cycling parameters set by Streck were used in each of the 30 amplification cycles. The resulting PCR Ct values are each associated with a specific fluorophore for each multiplexed target (e.g., HEX, FAM) and indicate the measurable outcomes for AMR genes.

Phenotypic Sample Processing

Targets for Bacterial Culturing Methods

Wastewater was plated to culture bacteria and selected for one of three different resistance targets: third-generation-cephalosporins-resistant bacteria, carbapenem-resistant bacteria, and fluoroquinolone-resistant bacteria. The primary bacteria that were targeted were *Escherichia coli* and *Klebsiella pneumoniae*, two members of Enterobacterales, bacteria that can cause infection and illness both in communities and healthcare settings (*ESBL-Producing Enterobacterales | HAI | CDC*, 2021). These targets were chosen to reflect organisms of critical concern as

determined by the US CDC critical priority targets for AMR genes published by the WHO, as well as the prevalence of AMR genes in Atlanta, Georgia, USA previously indicated by the CDC.

Third-generation cephalosporin (3GC) resistance - Cephalosporins are in the beta-lactam group of bactericidal antibiotics, and are produced through laboratory structural modifications. Cephalosporins are grouped by generations one through five, differentiated based on molecular structure and therapeutic function (Arumugham et al., 2023). The third-generation class of cephalosporins is most widely prescribed as a broad spectrum antibiotic used against both gram-positive and gram-negative organisms, and are particularly effective against beta-lactamases produced by *Klebsiella*, *Haemophilus influenzae*, and *E. coli*. Enterobacteriales like these can develop resistance to third generation cephalosporins (3GCs). 3GC resistance is mediated by extended-spectrum beta-lactamases (ESBLs), production of which by Enterobacteriales causes the breakdown of commonly used antibiotics, rendering antibiotic treatments like 3GCs useless against serious infections (*ESBL-Producing Enterobacterales | HAI | CDC*, 2021). Due to ESBL-production being the driving mechanism of 3GC-resistance, detected resistance to third-generation cephalosporins in this paper are presumed to be ESBL-producing bacteria.

Carbapenem-resistance (CR) - Carbapenem antibiotics are a potent group of antimicrobial agents that are used in treatment of patients with severe bacterial infections. Often these antibiotics are used for bacterial infections that have resistance to most other antimicrobial agents (Iovleva & Doi, 2017). The rise in prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE) highlights concerns for treatment options for severe bacterial

infections. There are three mechanisms that can lead to the development of CRE: high-level production of chromosomal AmpC cephalosporinase with impaired membrane permeability, production of carbapenemase, a β-lactamase that can hydrolyze carbapenems, or changes in the affinity of penicillin binding proteins (Yigit et al., 2001). Cabapenemase-producing *Enterobacteriaceae* (CPE) have been associated with serious infections including with *K. pneumoniae*, which can easily spread among patients and is difficult to treat. Production of carbapenemases by these pathogen strains will at minimum, reduce their susceptibility to carbapenem antibiotics, but more often causes total resistance to carbapenems (Iovleva & Doi, 2017).

Fluoroquinolone (FQ) resistance -Fluoroquinolones are broad spectrum antibiotics used in both human and veterinary medicine to treat bacterial infections (Piddock, 1998). In gram-negative bacteria (e.g., *E. coli*), the two enzyme targets are primarily DNA gyrase and secondarily DNA topisomerase IV, while in gram-positive bacteria (e.g., *Staphylococcus aureus*) the primary enzyme target is DNA topisomerase IV, and the secondary target is DNA gyrase. FQ resistance commonly results from mutation(s) in a small section of the enzyme subunits *gyrA* or *parC* (with rare mutations in gyrB or parE). Other genes that affect uptake of pharmaceuticals into bacterial cells can be overexpressed, increasing the secretion of quinolones from bacterial cells. This can lead to increases in the minimum inhibitory concentration (MIC) of antimicrobial agents including FQs. higher MIC values imply a reduced effectiveness of antimicrobial agents due to an increase in the amount of drug needed to inhibit bacterial growth (*13.5A*, 2018).

Bacterial Culture & Membrane Filtration Methods

Ms. Oluwatosin Olojo tested all samples for 3GC-resistant, carbapenem-resistant, and fluoroquinolone-resistant *Enterobacteriaceae* via membrane filtration using protocols developed under the supervision of Dr. Maya Nadimpalli. Briefly, serial dilutions of each sample were prepared in Phosphate Buffer Saline (PBS) (10^{-1}, 10^{-2}, 10^{-3}), then filtered through a 0.45um filter on a vacuum manifold in duplicate. Filters were plated on three types of selective media prepared in-house: (1) MacConkey agar supplemented with 4 µg/ml of ciprofloxacin for detection of fluoroquinolone-resistant bacteria, (2) CHROMagar ESBL for detection of 3GC-resistant bacteria, and (3) CHROMagar mSuperCARBA for detection of carbapenem-resistant bacteria. These agar plates were incubated for 16 to 24 hours. After incubation, colony-forming units (CFUs) on each plate were counted. For CHROMagar media, observed pink colonies–signifying *E. coli*–and blue colonies–signifying *Klebsiella, Enterobacter,* and *Citrobacter* (KEC) species–were counted.

Lactose fermentation produces lactic acid, which when plated on MacConkey agar, decreases the pH of the agar. This causes the pH indicator in the agar to turn pink, and colonies of lactase-producing-gram-negative bacteria will therefore present as pink on the MacConkey agar (Jung & Hoilat, 2023), while colonies of non-lactase-producing-gram-negative bacteria will appear as tan or opaque with no pH changes. Examples of gram-negative bacteria with lactose fermentation include *E. coli, Enterobacteria, and Klebsiella*. Examples of gram-negative bacteria without lactose fermentation include *Salmonella, Proteus, Yersinia, Pseudomonas*. However, *E. coli* can be present without lactose fermentation due to a lack of an enzyme lactose permease encoded by the lacY gene (Mazumder et al., 2022). This means that *E. coli* colonies on MacConkey agar can

present as either pink or tan colonies. Both pink and tan colonies were counted, typically representing *E. coli* and for the purposes of this analysis were treated together without differentiation between lactase-producing and non-lactase-producing gram-negative bacteria.

Plates within the countable range of 25-250 CFUs were prioritized, and the dilution which consistently produced these results was chosen for each target. The average count of CFUs for duplicates of each of the five media/outcome combinations were transformed into a value representing the estimated CFU/100mL in the original sample and used as continuous outcome variables in this analysis. The reported CFU was an average of two duplicates of the same sample and dilution level.

Demographic variables and population dataset

2020 U.S. American Community Survey

It was necessary to utilize existing community-level data to analyze the characteristics of populations living within the diverse communities reached by the target water plants. The U.S. Census Bureau's American Community Survey (ACS) most recent 2020 dataset was used. ACS is a nationwide survey that provides detailed population and housing information about communities yearly (Bureau, n.d.-a). This includes information on employment, occupations, educational attainment, veterans, homeowners, and much more with a vast library of variables related to social, economic, housing, and demographic characteristics of the population. Annually, the Census Bureau contacts households to gather information which is utilized to study changes in communities over time and inform public resource allocation. Given that ACS datasets are comprehensive and that data was available for the sewershed areas of the wastewater

treatment plants in metropolitan Atlanta, this dataset was a sensible source of social determinants of health variables within metropolitan Atlanta.

ACS census variables correspondence to AMR target risk factors

ACS variables of interest were isolated based on previous knowledge regarding the relationship between these factors and antibiotic prescriptions, as well as the relationship between antibiotic use and AMR prevalence.

Age & Sex - Previous studies have cited that populations with higher rates of antibiotic prescriptions were more female (Schröder et al., 2016) and older populations (Kabbani et al., 2018). Another study found that U.S. counties with more obese persons, infants, and children less than or equal to 2 years old, were more likely to have high prescription rates (Hicks et al., 2015). Differences in antibiotic prescription rates associated with patient gender and age raise interest in exploring the relationship between these demographic characteristics and environmental AMR prevalence (Kabbani et al., 2018).

Access to Healthcare - Access to healthcare in communities is linked to several factors, including affordability and access to health insurance, income barriers-including low income status, and in the context of healthcare settings, prescriptions, and other resources (National Academies of Sciences et al., 2018). Several of these factors have been linked to increasing or decreasing antibiotic use. In one study, inappropriate antibiotic treatments were seen more frequently in those with Medicare, Medicaid, and no insurance compared to those with commercial insurance (McHale et al., 2020). Inappropriate treatments included the lack of proper

treatment regimen adherence, as well as use of leftover antibiotics improperly. An article published in *The Lancet Global Health* found that among the study population, those not experiencing poverty were more likely to misuse antibiotics than those in poverty (Obua et al., 2023), while past articles highlight poverty as a driver for antibiotic misuse and increases in AMR. Given preliminary evidence of potential relationships between AMR or mechanisms leading to AMR and variables related to access to healthcare, several prevalent proxy variables were included in this analysis - median household income, the percentage of individuals living below the poverty line, and the percentage of a population that is without health insurance.

Foreign-Born - A significant driver for rises in AMR prevalence is increased global connectivity and ease of human travel. To analyze this factor as it relates to AMR prevalence at the community-level, a proxy variable was used to represent potential increases in travel activity to other areas of the world outside of the United States: foreign-born status. This variable accounts for the status of foreign born as any individual in the population who was not born in the United States, including those who were naturalized and are U.S. citizens but were not born in the United States (Bureau, n.d.-b). Use of this variable to represent frequency of travel requires the assumption that those who are foreign-born will be more likely to travel outside of the U.S. or receive visitors from outside the US than those who were born in the United States.

Race - Previous studies have found disparities in antimicrobial agent acquisition in populations based on race. In 2014-205, it was found that White persons reported twice the antibiotic prescription fills per capita compared to other races and ethnicities (Olesen & Grad, 2018). These disparities were seen across multiple classes of antimicrobial agent prescription fills. While there

are limitations to using prescription fills as proxies for antibiotics use, established correlations raise interest in exploring a more direct relationship between AMR prevalence and racial distribution in diverse communities. Non-prescription antibiotic use is one aspect of antibiotic use and AMR risk factors that is left out when focusing on prescription fills. Previous qualitative research has indicated that Hispanic individuals were nearly three times as likely to report non-prescription antibiotic use compared to Non-Hispanics (NHs). Other studies found that compared to Non-Hispanic Whites, there was less antibiotic use knowledge, acquisition of non-prescription antibiotics, and dissatisfaction with lack of antibiotic prescription for treatment (*Knowledge and Attitudes Regarding Antibiotic Use Among Adult Consumers, Adult Hispanic Consumers, and Health Care Providers — United States, 2012–2013*, n.d.). Demographic data was collected for percentage of the racial distribution of a population that were Pacific Islander or Native American. However, they are not focused on in this paper for any findings drawn from statistical analysis due to the extremely small percentages which they represent in comparison to other variables.

Variable	Census Geo_ID	Description of the Variable
Foreign Born Status	DP02_0094E	Estimate of foreign-born population among total population surveyed for place of birth.
Percentage Female	DP05_0003E	Estimate of female population among total population surveyed for sex and age.
Percentage Aged Under 5 years old	DP05_0005E	Estimate of under 5 years old population among total population surveyed for sex and age.
Percentage Aged Over 75 years old	DP05_0016E + DP05_0017E	Sum of the estimate of between 75 to 84 years old population among total population surveyed for sex and age and the estimate of over 85 years old population among total population surveyed for sex and age.
Percentage	DP03_0099E	Estimate of no health insurance coverage population

Uninsured		among total civilian noninstitutionalized population responding about health insurance coverage.
Median Household Income	DP03_0062E	Estimate of median household income (in inflation-adjusted USD) among total households with measures for income and benefits.
Percentage Below the Poverty Line	DP03_0128PE	Percentage of all peoples whose income in the past 12 months was below the poverty line. (in inflation-adjusted USD) among total households with measures for income and benefits.
Percentage of Hispanics	DP05_0071E	Estimate of Hispanic or Latino people of any race among the total population.
Percentage of Non-Hispanic Blacks	DP05_0078E	Estimate of Non-Hispanic Black or African American people of any race among the total population.
Percentage of Non-Hispanic Whites	DP05_0077E	Estimate of Non-Hispanic White people of any race among the total population.
Percentage of Non-Hispanic Asians	DP05_0080E	Estimate of Non-Hispanic Asian people of any race among the total population.
Percentage of Non-Hispanic Native Americans	DP05_0079E	Estimate of Non-Hispanic Native American tribe people of any race among the total population.
Percentage of Non-Hispanic Pacific Islanders	DP05_0081E	Estimate of Non-Hispanic Pacific-islander American tribe people of any race. This includes native Hawaiian and other Pacific Islanders.

Table 1. American Community Survey (ACS) variables used in this analysis.

Data Analysis

Analysis of the AMR culture-based outcomes and Census-derived variables was run using R Studio Version 4.2.2. Both outcomes and exposures are continuous variables in this cross-sectional study, which are appropriate for conducting a linear regression for five AMR bacterial culture outcomes: *E. coli* outcomes in 3GC-resistant and carbapenem-resistant media, KEC

outcomes in 3GC-resistant and carbapenem-resistant media, and gram-negative bacteria in FQ-resistant media.

Outcome Measure

Outcomes were analyzed for the colonies grown from filters of the most concentrated dilution of the samples. Out of the three-dilution series, the concentrations used in this analysis were the ones that yielded the most countable results in the dilution series, having more outcomes that fell within the target ranges for colony counting (20 - 150 CFU/mL). For this reason, the 10^{-1} dilution was used in testing for 3GC-resistance and carbapenem-resistance, and the 10^{-2} dilution was used for testing for FQ resistance.

Census Variable Data Cleaning

To conduct an analysis of the wastewater treatment plant data, the census data was aggregated in summary statistics for the respective catchment areas of the treatment plants. The summary statistics were weighted to account for the percentage of the given census tracts that were within a given catchment area. The assignment of a geographic area in each census tract covered by a treatment plant was provided by Stephen Mugel by utilizing catchment area shape files.

Simple Linear Regression

Simple linear regression models for individual exposure variables were used to test for correlations between demographics and AMR prevalence. There were several assumptions made in conducting simple linear regression models: homogeneity of variance, independence between observations, and normality. Specifically, it is assumed that the SLR error magnitude does not

significantly change between values of the exposure variable of interest, that each WWTP observation is independent, and that the data does not violate the assumption of normality. The normality of the dependent variables was assessed using a visual assessment of Q-Q plots. The exposure variables that were measured included percentages of those communities that were female, foreign-born, uninsured, below the poverty level, age less than five years old, and age greater than 75 years old, as well as the median household income and racial distribution for each sewershed area.

Multiple Linear Regression Model

A multiple linear regression (MLR) was used to understand the complex contributions of several risk factors identified in the census data as they exist in one model. Although the sample size used here is smaller than the often-accepted limit of 10, smaller sample sizes are sometimes used and an MLR model was used here with the understanding of the limitation smaller sample sizes creates on the generalizability of conclusions. This was an accepted trade off given the preliminary nature of this paper and the purpose in using these results to shape other research studies. To use an MLR model, several assumptions were made. This includes assumptions that there is homogeneity of variance, no multicollinearity, independence between observations, normality in the data distribution, and linearity in the dataset. To meet the multicollinearity assumption, tests for collinearity were conducted on a full multiple linear regression model which included the following variables: median household income, percentage of the population under the poverty line, uninsured, and percentage with a foreign-born status to obtain variance inflation factor (VIF) values. Due to the consistency of census tract data values associated with all five outcomes for each respective wastewater plant site, a test for collinearity was run on one

outcome with the assumption that collinearity results will be identical for the remaining five outcomes. Results from the test for collinearity were used to decide which variables remained or were removed in the final adjusted multiple linear regression model. VIF values greater than 10 were considered for removal, prioritizing the removal of variables with higher VIF values (Bhandari, 2020). The reduced model was then used to run a multiple linear regression model in R Studio for each of the five outcomes, and the resulting estimates, standard errors, and p-values were reported.

3 RESULTS

Demographics

Census tract data aggregated to each WWTP sewershed area are included in Table 2. The communities included across all sewershed areas for the WWTPs were on average, 51.6% female, with 12.3% of the population being born outside of the United States, 9.4% of the population being uninsured, and 12.4% of the population living below the poverty line. The total population had an average median household income of $94,704.20 USD. Among the sewershed areas, the lack of insurance coverage ranged from 5.0% to 15.0%, the percent female ranged from 49.4% to 54.6%, the percent foreign-born ranged from 3.8% to 13.2%, poverty status ranged from 3.1 % to 27.1% and median household income ranged from $41,518,79 to $158,895.40 USD.

The reported racial distribution indicated a majority of the total aggregated population was Non-Hispanic (NH) White or Black. However, there are drastically different racial distributions between each treatment plant-specific aggregated Census data. Across the census tracts of communities feeding into the wastewater treatment plants, a majority of the populations was in either the Non-Hispanic White (43%) or Non-Hispanic Black (40%) racial categories, with smaller percentages of Hispanic (6%), Non-Hispanic Asian (7%), Non-Hispanic Native American (<1%) and Non-Hispanic Pacific Islander (<1%). Racial distribution varied greatly across the different wastewater treatment plants, with the distribution of Hispanic ranging from 3% to 13%, Non-Hispanic White ranging from 5% to 80%, Non-Hispanic Black ranging from 8% to 89%, and Non-Hispanic Asian ranging from 7% to 24%.

Wastewater Treatment Plants (WWTPs)	Average across all WWTPs	Big Creek (BIG)	Camp Creek Basin (CMP)	Johns Creak (JON)	Little River (LIR)	RM Clayton (RMC)	South River (SOR)	Utoy Creek (UTC)
Median Household Income (USD) (mu)	$94,704.20	$112,122.00	$60,568.57	$128,196.70	$158,895.40	$105,836.60	$55,791.44	$41,518.79
Sex (%)								
Female	51.6	50.3	54.6	50.8	49.4	50.6	53.1	52.1
Insurance Coverage (%)								
No	9.35	9.76	11.09	6.16	5.04	5.17	13.21	14.99
Foreign Born Status (%)								
Yes	12.28	25.91	5.78	28.87	5.60	11.45	4.54	3.80
Poverty Status (%)								
In Poverty	12.42	6.31	11.98	4.26	3.10	11.75	22.46	27.05
Race (%)								
Hispanic	5.96	12.77	4.03	6.49	4.82	6.00	4.40	3.17
NH White	43.22	53.81	4.86	56.19	79.49	69.09	31.30	7.80
NH Black	40.02	14.59	88.98	10.06	7.76	15.35	58.52	84.90
NH Asian	7.31	13.63	0.363	23.65	3.06	6.31	2.40	1.74
NH Native American	0.14	0.15	0.042	0.26	0.021	0.12	0.26	0.16
NH Pacific Islander	0.036	0.022	0.080	0	0.085	0.059	0	0.0038

Table 2. Demographic characteristics of Atlanta metro communities taken from census tracts aggregated by wastewater treatment plants.

The census age categories (Figure 2) indicate that there are differences in the distribution of age in different sewershed areas. There is the widest variety in the percentage of those aged 25-34 in different sewershed areas (5% to 26.5%). There was a smaller range for the percentage of those aged less than 5 ranging from 4.4% to 8.0%.

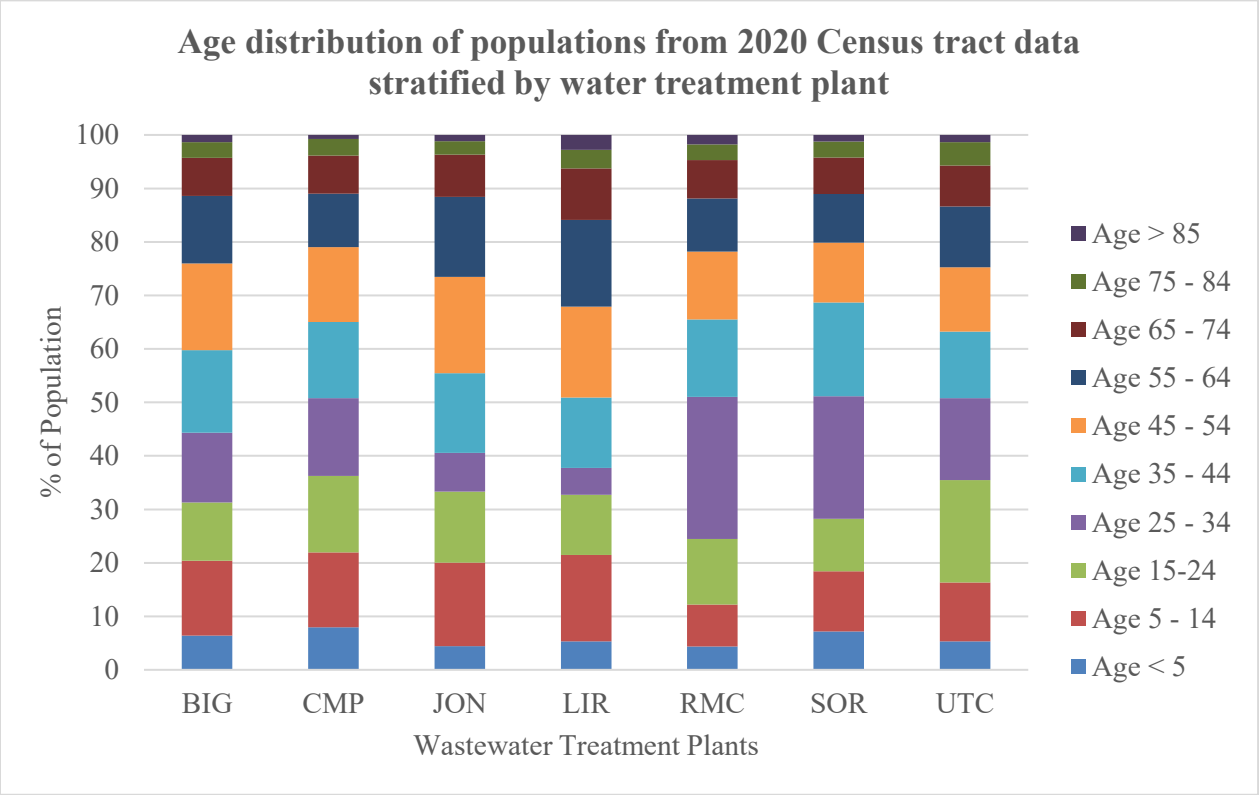

Figure 2. Graphical distribution of age distribution of populations within each sewershed area of the respective wastewater treatment plants using Census data.

AMR	3GC-resistant		Carbapenem-resistant		FQ-resistant
Bacterial Outcome	***E. coli***	**KEC**	***E. coli***	**KEC**	**Lac Pos and Neg**
	mean CFU/100 mL (pink colonies)	**mean CFU/100 mL (blue colonies)**	**mean CFU/100 mL (pink colonies)**	**mean CFU/100 mL (blue colonies)**	**mean CFU/100 mL (pink + tan colonies)**
Average Across all WWTPs	6.2	24.9	75.9	114	3.3
Wastewater Treatment Plant					
Big Creek (BIG)	6	34	141	125.5	4.5
Camp Creek Basin (CMP)	1	1	67.5	169	5.1
Johns Creak (JON)	9.5	34	157.5	157	6.45
Little River (LIR)	0	0	1.5	4.5	0
RM Clayton (RMC)	27	103.5	77	207	3.2
South River (SOR)	0	1	81	131	3.8
Utoy Creek (UTC)	0	0.5	6	4.5	0.3

Table 3. Bacterial culturing outcomes associated with seven wastewater treatment plants (WWTPs) in metropolitan Atlanta, including five outcomes: 3GC-resistance in *E. coli* and KEC species, carbapenem-resistance in *E. coli* and KEC species, and FQ-resistance in gram-negative bacteria (with and without lactose fermentation).

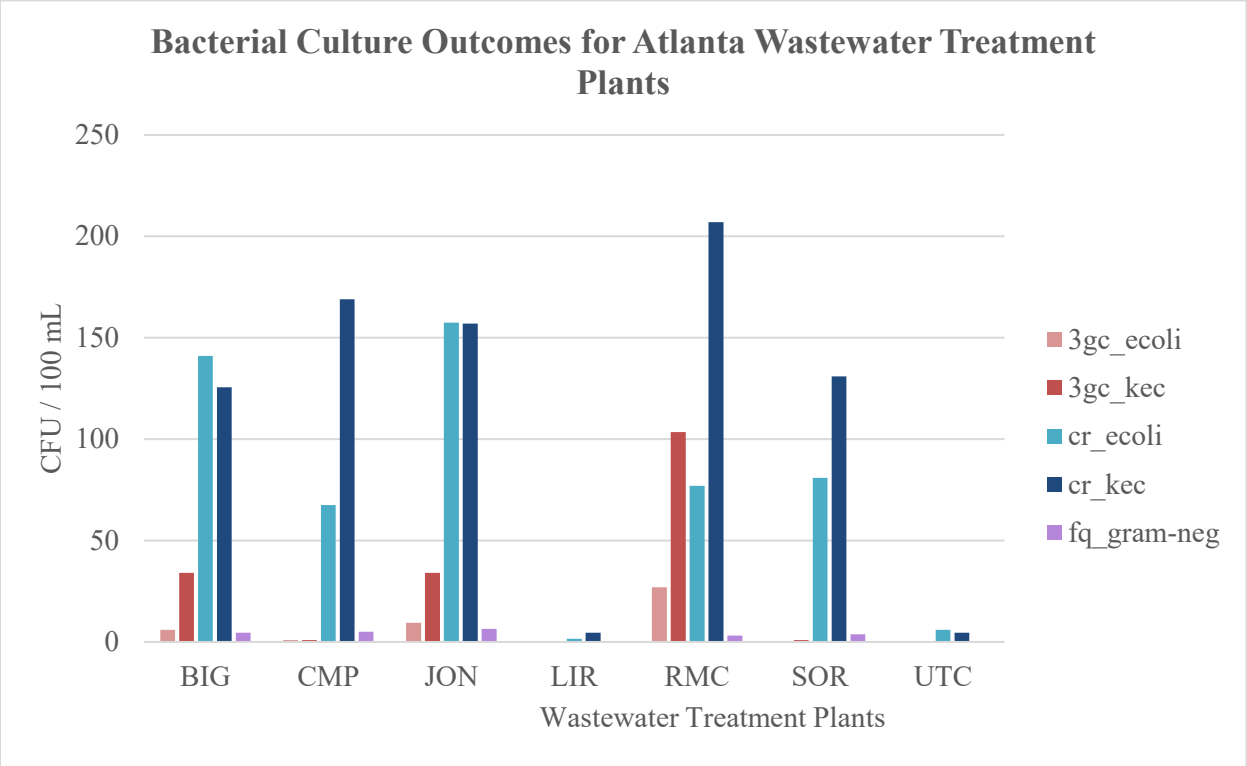

Figure 3. Bacterial culture colony count outcomes for seven wastewater treatment plants

Simple Linear Regression

Normality

The Q-Q plots (Figure 3) for the six outcome variables confirmed the normality assumption for this dataset. There is one residual value for each *E. coli* and KEC species with 3GC-resistance, which deviates from the expected observed/predicted range. However, due to the small sample size of this dataset and the observed normality in the rest of the data points, normality is assumed for these two outcome variables alongside the other four outcome variables.

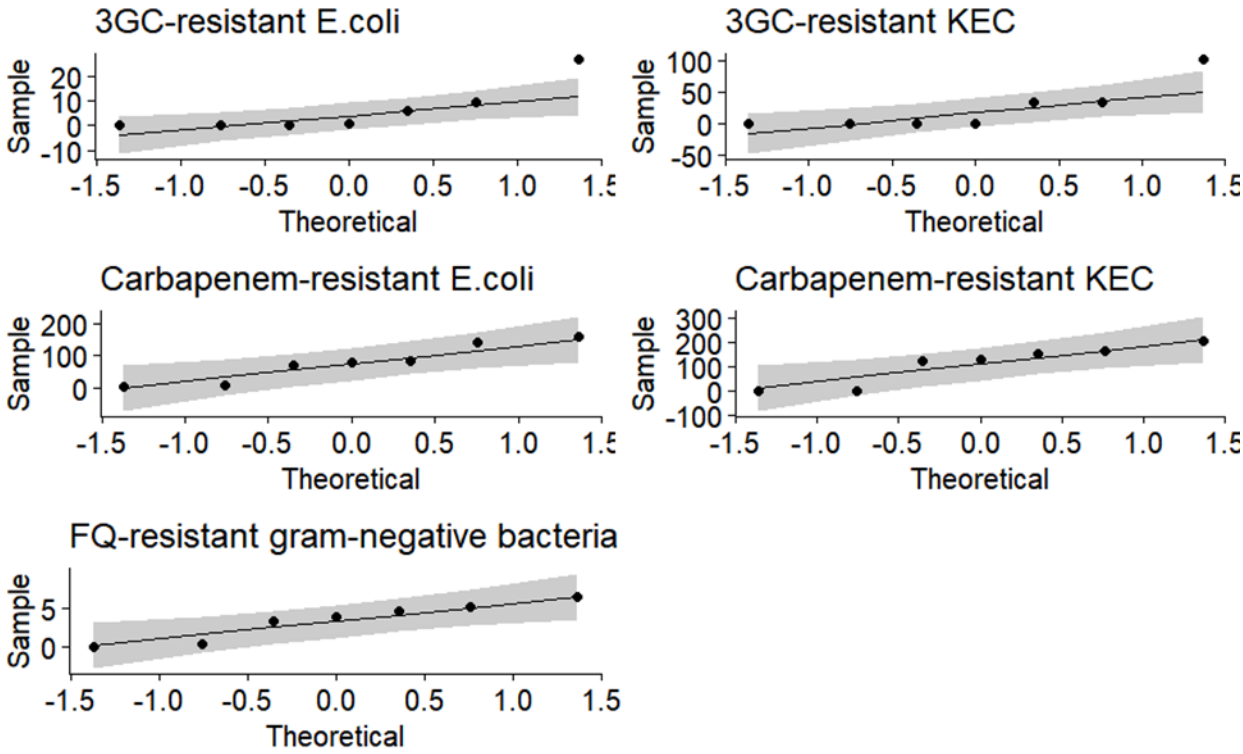

Figure 4. Q-Q Plots showing observed and predicted residual values for the five outcome variables testing for normality.

SLR Models: Foreign-Born Variable

The simple linear regression models for 3GC-resistance (Table 4a), carbapenem-resistance (Table 4b), and FQ-resistance (Table 4c) showed a 0.31-CFU/100mL increase in 3GC-resistant *E. coli*, a 1.36-unit increase in 3GC-resistant KEC species colonies, a 4.9-unit increase in carbapenem-resistant *E. coli* colonies, a 2.9-unit increase in carbapenem-resistant KEC species colonies, and a 0.15-unit increase in FQ-resistant gram-negative bacteria with and without lactose fermentation for every 1% increase in the percentage of the population that is foreign-born. The only significant linear relationship was between increase in carbapenem-resistant *E. coli* and percent foreign-born.

SLR Models: Sex Variable

There were no significant linear relationships observed between AMR outcomes and percent female. Although not significant, the model showed a 1.85-unit decrease in 3GC-resistant *E. coli* colonies, an 8.06-unit decrease in 3GC-resistant KEC species colonies, a 2.12-unit decrease in carbapenem-resistant *E. coli* colonies, a 12-unit increase in carbapenem-resistant KEC species colonies, and a 0.40-unit increase in FQ-resistant gram-negative bacteria with and without lactose fermentation for every 1% increase in the percentage of the population that is female.

SLR Models: Age Variable

There were no significant linear relationships observed between the percentage of the civilian population under 5 years old and respective 3GC-resistant, CR, and FQ-resistance. Although not significant, models showed a 4.3-unit decrease in 3GC-resistant *E. coli*, a 16.0-unit decrease in 3GC-resistant KEC species, a 1.2-unit decrease in carbapenem-resistant *E. coli*, a 5.7-CFU/100mL increase in carbapenem-resistant KEC species, and a 0.38-CFU/100mL increase in FQ-resistant gram-negative bacteria with every 1% increase in the population under 5 years old ($p > 0.05$).

There were significant negative linear relationships observed between the percentage of the civilian population over 75 years old and respective CR and FQ-resistance, with a 52-unit decrease in carbapenem-resistant *E. coli*, a 68-unit decrease in carbapenem-resistant KEC species, and a 2.4-unit decrease in FQ-resistant gram-negative bacteria with every 1% increase in the population over 75 years old ($p < 0.05$). There were no significant linear relationships observed between the percentage of the civilian population over 75 years old and 3GC-resistant *E. coli* and KEC species; models showed a non-significant 2.0-unit decrease in 3GC-resistant *E.*

coli and a 7.7-unit decrease in 3GC-resistant KEC species with every 1% increase in the population over 75 years old ($p > 0.05$).

SLR Model: Median Household Income Variable

Rises in median household income in the population had no significant linear relationships with increases in CFU/100 mL of 3GC resistance in *E. coli* and KEC species and CR in *E. coli.* There were also no significant linear relationships between CR in KEC species and FQ-resistance in gram-negative bacteria and increases in median household income. These findings were extremely small in magnitude and given the non-significant p-value and collinearity between median household income, poverty, and uninsured, median household income was not a priority for analysis.

SLR Model: Poverty Variable

There were no significant linear relationships between the percentage of population below the national poverty line and AMR outcomes. Although not significant, models indicated a 0.26-CFU/100mL decrease in 3GC-resistant *E. coli*, a 1.1-CFU/100mL decrease in 3GC-resistant KEC species, a 2.6-CFU/100mL decrease in carbapenem-resistant *E. coli*, a 1.8-CFU/100mL decrease in carbapenem-resistant KEC species, and a 0.083 CFU/100mL decrease in gram-negative bacteria for every 1% increase in the populations living below the national poverty line depending on household status.

SLR Model: Uninsured Variable

While there were no significant linear relationships between percentage uninsured and AMR outcomes, models indicated a 1.4-CFU/100mL decrease in 3GC-resistant *E. coli*, a 5.4-

CFU/100mL decrease in 3GC-resistant KEC species, a 3.2- CFU/100mL decrease in carbapenem-resistant *E. coli*, a 5.2-CFU/100mL decrease in carbapenem-resistant KEC species, and a 0.07-CFU/100mL decrease in gram-negative bacteria for every 1% increase in the population uninsured ($p > 0.05$ each).

SLR Model: Race Variables

Hispanic: There were no significant linear relationships observed between each outcome and Hispanic percentage. However, models indicated a 0.73-CFU/100mL increase in 3GC-resistant *E. coli* colonies, an 4.0-CFU/100mL increase in 3GC-resistant KEC species colonies, a 12.5-CFU/100mL increase in carbapenem-resistant *E. coli* colonies, a 6.5-CFU/100mL increase in carbapenem-resistant KEC species colonies, a 0.29-CFU/100mL increase in FQ-resistant gram-negative bacteria for every 1% increase in the percentage of the population that is Hispanic.

Non-Hispanic Black: While no significant linear relationships were observed between each outcome and NH Black percentage, the model showed a 0.13-CFU/100mL decrease in 3GC-resistant *E. coli*, an 0.55-CFU/100mL decrease in 3GC-resistant KEC species, a 0.65-CFU/100mL decrease in carbapenem-resistant *E. coli* colonies, a 0.28-CFU/100mL decrease in carbapenem-resistant KEC species colonies, a 0.0067-CFU/100mL decrease in FQ-resistant gram-negative bacteria for every 1% decrease in the percentage of the population that is Non-Hispanic Black.

Non-Hispanic White: There were no significant linear relationships between NH White percentage of the population and AMR outcomes. Although not significant, models indicated a 0.17-CFU/100mL increase in 3GC-resistant *E. coli*, an 0.66-CFU/100mL increase in 3GC-resistant KEC species, a 0.35-CFU/100mL increase in carbapenem-resistant *E. coli* colonies, a

0.13-CFU/100mL decrease in carbapenem-resistant KEC species colonies, a 0.0073-CFU/100mL decrease in FQ-resistant gram-negative bacteria for every 1% decrease in the percentage of the population that is Non-Hispanic White.

Non-Hispanic Asian: There was a significant linear relationship between increase in CR *E. coli* and NH Asian percentage, with a 5.8-CFU/100mL increase in carbapenem-resistant *E. coli* for every 1% increase in percentage of the population. This was the only significant linear relationship; while not significant, other models showed a 0.37-CFU/100mL increase in 3GC-resistant *E. coli* colonies, a 1.5-CFU/100mL increase in 3GC-resistant KEC species colonies, a 3-CFU/100mL decrease in carbapenem-resistant KEC species, a 0.17-CFU/100mL decrease in FQ-resistant gram-negative bacteria for every 1% increase in the percentage of the NH Asian population.

Individual Simple Linear Regression Models: Demographic Risk Factors & 3GC resistance in *E. coli* and KEC (*Klebsiella, Enterobacter,* and *Citrobacter*) species						
Exposure Variables	***E. coli***			**KEC**		
	Estimate	**Standard Error**	**P-value**	**Estimate**	**Standard Error**	**P-value**
% foreign born	0.31	0.4	0.47	1.4	1.5	0.40
% female	-1.9	2	0.46	-8.1	9	0.39
% under 5 y.o.	-4.3	3	0.16	-16	10.	0.18
% above 75 y.o.	-2.0	4	0.67	-7.7	17	0.67
% uninsured	-1.4	0.9	0.17	-5.4	4	0.19
Median household income	6.2e-05	9e-05	0.52	2.5e-04	3e-04	0.50
% in poverty	-0.26	0.4	0.56	-1.1	1.6	0.54
Race						
% Hispanic	0.73	1.3	0.61	4.0	5	0.46
% NH Black	-0.13	0.10	0.27	-0.55	0.4	0.23
% NH White	0.17	0.13	0.27	0.66	0.5	0.24
% NH Asian	0.37	0.5	0.49	1.5	1.9	0.46

Table 4a. Simple linear regression model results between single exposure variables and CFU/100mL of 3GC resistance found in *E. coli* and KEC.

Individual Simple Linear Regression Models: Demographic Risk Factors & carbapenem resistance in *E. coli* and KEC (*Klebsiella*, *Enterobacter*, and *Citrobacter*) species						
Exposure Variables	***E. coli***			**KEC**		
	Estimate	**Standard Error**	**P-value**	**Estimate**	**Standard Error**	**P-value**
% foreign born	4.9	1.2	0.0097 *	2.9	3	0.39
% female	-2.2	15.	0.89	12.	19.	0.55
% under 5 y.o.	-1.2	20	0.95	5.7	30	0.83
% above 75 y.o.	-52	15.	0.017 *	-68	20	0.021 *
% uninsured	-3.2	7	0.65	-5.2	9	0.57
Median household income	2.6e-04	5e-04	0.66	-9.6e-05	7e-04	0.90
% in poverty	-2.6	2	0.32	-1.8	3	0.62
Race						
% Hispanic	13.	6	0.098	6.5	11.	0.57
% NH Black	-0.65	0.7	0.38	-0.28	1.0	0.78
% NH White	0.35	0.9	0.71	0.13	1.2	0.92
% NH Asian	5.8	2	0.024 *	3.0	4	0.48

Table 4b. Simple linear regression model results between single exposure variables and CFU/100mL of carbapenem-resistance found in *E. coli* and KEC. Note: * = significant p-value ($|p| < 0.05$)

Individual Simple Linear Regression Models: Demographic Risk Factors & FQ resistance in gram-negative bacteria with and without lactase fermentation			
Exposure Variables	**Estimate**	**Standard Error**	**P-value**
% foreign born	0.15	0.08	0.11
% female	0.40	0.6	0.51
% under 5 y.o.	0.38	0.8	0.65
% above 75 y.o.	-2.4	0.2	0.00016 *
% uninsured	-0.07	0.3	0.80
Median household income	-7.1e-07	2.5e-05	0.98
% in poverty	-0.083	0.11	-0.49
Race			
% Hispanic	0.29	0.3	0.40
% NH Black	-0.0067	0.03	0.83
% NH White	-0.0073	0.04	0.85
% NH Asian	0.17	0.1	0.14

Table 4c. Simple linear regression model results between single exposure variables and CFU/100mL of FQ resistance found in gram-negative bacteria with and without lactose fermentation. Note: * = significant p-value ($|p| < 0.05$)

Multiple Linear Regression Modeling

To account for the intersection between exposures, a multiple variable analysis was run using R Studio following test for collinearity on a full model with the following variables: foreign_born_pct, female_pct, agelt5_pct, age_gt_75, uninsured_pct, median_hh_income, poverty_pct. Testing for collinearity revealed collinearity between the model exposures and poverty, median household income, and uninsured percentage using VIF values. The model was rerun after removing each of these three variables separately and ran again removing both poverty and median household income. Due to the interest in the analysis of uninsured percentages as a proxy variable for access to healthcare, poverty, and median household income were removed from the final multiple linear regression model (Equation 1). There was additional collinearity noted between the percent of the population aged greater than 75 years old and the female percentage. For this reason, the percentage of the population aged greater than 75 years old was removed from the final model (Equation 1). The remaining variables in the reduced model were uninsured, foreign-born, female, and aged less than five years old.

$$reduced\ model: CFU/100ml_{X-resistance} = \beta_0 + \beta_1 uninsured + \beta_2 foreign\_born + \beta_3 female + \beta_4 agelt5$$

Equation 1. The reduced model was used in the multiple linear regression for all five outcomes.

Tests for Collinearity

Model	% Uninsured		% Female		% Foreign Born		Median Household Income		% in Poverty		% Age Less than 5 y.o.		% Age Greater than 75 y.o.	
	In Model?	VIF	In Model?	VIF	In Model?	VIF	In Model?	VIF	In Model?	VIF	In Model?	VIF	In Model?	VIF
1	Yes	14.1	Yes	14.2	Yes	4.5	Yes	53.1	Yes	39.4	Yes	7.04	No	n/a
2	No	n/a	Yes	29	Yes	13.2	Yes	59	Yes	33.9	Yes	2.9	Yes	9.4
3	Yes	49.8	No	n/a	Yes	24.9	Yes	31.6	Yes	37	Yes	19.6	Yes	16.2
4	Yes	11.2	Yes	12.3	No	n/a	Yes	47.1	Yes	20.4	Yes	4.9	Yes	2.5
5	Yes	340	Yes	106	Yes	318	No	n/a	Yes	274	Yes	105	Yes	202
6	Yes	23.6	Yes	15.1	Yes	16.7	Yes	33.2	No	n/a	Yes	6.8	Yes	18.3
7	Yes	8.6	Yes	33.6	Yes	17.0	Yes	54.2	Yes	29.0	No	n/a	Yes	13.9
8	Yes	2.9	Yes	14.1	Yes	10.1	No	n/a	No	n/a	Yes	2.35	Yes	11.7
9	Yes	2.8	Yes	14.0	Yes	9.8	No	n/a	No	n/a	No	n/a	Yes	11.3
10	**Yes**	**1.9**	**Yes**	**2.9**	**Yes**	**1.3**	**No**	**n/a**	**No**	**n/a**	**Yes**	**2.3**	**No**	**n/a**

Table 5. Tests for collinearity in a multiple linear regression model with 7 variables for each of the five continuous outcomes.

3GC-resistance

In the MLR analysis of samples from seven wastewater treatment plants, there were no significant linear relationships found between the frequency of female and the CFU/100mL of 3GC resistance in both *E. coli* and KEC species and the frequency of foreign-born and the CFU/100mL of 3GC resistance in both *E. coli* and KEC species ($p > 0.05$ for each). Although there were no significant linear relationships, the model showed a 3.1-CFU/100mL increase (± 4.4) in the CFU/100mL of 3GC-resistant *E. coli* and an 8.6-CFU/100mL increase (± 18.0) in the CFU/100mL of 3GC-resistant KEC species for every 1% increase in female percentage. There was a 0.13-CFU/100mL increase (± 0.5) in the CFU/100mL of 3GC-resistant *E. coli* and a 0.66-CFU/100mL increase (± 2.1) in the CFU/100mL of 3GC-resistant KEC species for every 1% increase in foreign-born.

There are no significant linear relationships found between the frequency of uninsured and the CFU/100mL of 3GC resistance or between the frequency of under 5 years old and the CFU/100mL of 3GC resistance in either *E. coli* or KEC species. Although there lacked any significant linear relationship, the models showed a 1.3-CFU/100mL decrease in the CFU/100mL of 3GC-resistant *E. coli* and a 4.2-CFU/100mL decrease in the CFU/100mL of 3GC-resistant KEC species for every 1% increase in uninsured percentage. Models also showed a 4.7-CFU/100mL decrease in the CFU/100mL of 3GC-resistant *E. coli* and a 15.5-CFU/100mL decrease in the CFU/100mL of 3GC-resistant KEC species for every 1% increase in under 5 years old percentage.

Carbapenem-resistance

In the MLR analysis of samples from seven wastewater treatment plants, there were no significant linear relationships found between the percentage of female and the CFU/100mL of carbapenem-resistance or the percentage of foreign-born and the CFU/100mL of carbapenem-resistance in both *E. coli* and KEC species ($p > 0.05$ for each). Although there were no significant linear relationships, the models showed a 12.7-CFU/100mL increase (± 12.9) in the CFU/100mL of carbapenem-resistant *E. coli* and a 45.8-CFU/100mL increase (± 30) in the CFU/100mL of carbapenem-resistant KEC species for every 1% increase in female percentage. Models also indicated a 5.8-CFU/100mL increase (± 1.4) in the CFU/100mL of carbapenem - resistant *E. coli* and a 4.0-CFU/100mL increase (± 3.4) in the CFU/100mL of carbapenem-resistant KEC species for every 1% increase in foreign-born.

There were no significant linear relationships found between the frequency of uninsured and the CFU/100mL of carbapenem-resistance in both *E. coli* and KEC species. Despite lacking any

significant linear relationships, the models showed a 1.8-CFU/100mL decrease (± 4.5) in the CFU/100mL of carbapenem-resistant *E. coli* and a 14.0-CFU/100mL decrease (± 11.2) in the CFU/100mL of carbapenem-resistant KEC species for every 1% increase in uninsured percentage. There were also no significant linear relationships found between the frequency of percentage less than five years old and the CFU/100mL of carbapenem-resistance in both *E. coli* and KEC species. Although there were no significant linear relationships, the models indicated a 5.17-CFU/100mL increase (± 14.0) in the CFU/100mL of carbapenem-resistant *E. coli* and a 4.72-CFU/100mL decrease (± 35.6) in the CFU/100mL of carbapenem-resistant in KEC species for every 1% increase in under 5 years old percentage.

FQ-resistance

In the MLR analysis of samples from seven wastewater treatment plants, there was a significant positive linear relationship found between frequency of foreign-born and the CFU/100mL of FQ-resistance gram-negative bacteria ($p < 0.05$ for each). Specifically, there was a 0.21-CFU/100mL increase (± 0.04) in the CFU/100mL of FQ-resistant gram-negative bacteria for every 1% increase in foreign-born. This was the only significant linear relationship, however, the models showed a 1.2-CFU/100mL increase (± 0.3) in FQ-resistant gram-negative bacteria for every 1% increase in percent female, a 0.17-CFU/100mL increase (± 0.4) in the CFU/100mL of FQ-resistant gram-negative bacteria for every 1% increase in under 5 years old percentage, and a 0.25-CFU/100mL decrease (± 0.1) in the CFU/100mL of FQ-resistant gram-negative bacteria for every 1% increase in uninsured percentage.

Bacteria	*E. coli*			KEC (*Klebsiella, Enterobacter,* and *Citrobacter)*		
Exposure Variable	**Estimate**	**Standard Error**	**P-value**	**Estimate**	**Standard Error**	**P-value**
% Uninsured	-1.3	1.6262	0.5	-4.21	6.7	0.595
% female	3.06	4.3765	0.56	8.55	18.0	0.683
% foreign born	0.13	0.4967	0.8	0.66	2.1	0.777
% under 5 y.o.	-4.74	5.1684	0.5	-15.48	21.4	0.544

Table 6a. Results from two multiple linear regression models for *E. coli* and KEC species selected for resistance to 3rd generation cephalosporins (3GCs).

Bacteria	*E. coli*			KEC (*Klebsiella, Enterobacter,* and *Citrobacter)*		
Exposure Variable	**Estimate**	**Standard Error**	**P-value**	**Estimate**	**Standard Error**	**P-value**
% Uninsured	-1.795	4.5	0.7258	-14.017	11.2	0.337
% female	12.735	12.0	0.3991	45.790	30.1	0.268
% foreign born	5.833	1.4	0.0503	3.985	3.4	0.364
% under 5 y.o.	5.165	14.15	0.7500	-4.715	35.6	0.907

Table 6b. Results from two multiple linear regression models for *E. coli* and KEC species selected for resistance to carbapenems.

Bacteria	Gram-negative bacteria with and without lactase fermentation		
Exposure Variable	**Estimate**	**Standard Error**	**P-value**
% Uninsured	-0.25	0.1	0.19
% female	1.2	0.3	0.073
% foreign born	0.21	0.04	0.034 *
% under 5 y.o.	0.17	0.4	0.72

Table 6c. Results from one multiple linear regression model for gram-negative bacteria with and without lactase fermentation selected for resistance to fluoroquinolones (FQ). Note: * = significant p-value ($|p| < 0.05$)

4 DISCUSSION

Interpretation of Findings

There has been a dearth of studies understanding how antimicrobial resistance prevalence relates to social determinants of health and this research aimed to improve the identification of risk factors for further investigation. Overall, the results from this study show that multiple types of antimicrobial resistant bacteria are abundant and can be cultured readily from wastewater. Carbapenem-resistant bacteria were the most prevalent of the targets cultured in this study, and FQ-resistant gram-negative bacteria was least prevalent in these samples. Although the sample size for this study was limited, the regression analysis suggests that there are significant linear relationships between several demographic factors and carbapenem and fluoroquinolone resistance and further areas that although not significant warrant further investigation.

Increases in AMR prevalence

The relationship between increased FQ resistance in gram-negative bacteria and a higher percentage of the population identifying as foreign born was the only significant positive correlation found for the percentage foreign born. These results are consistent with previous findings relating global connectivity resulting in greater long-distance AMR pathogen transmission, as well as variability in global antibiotic stewardship programs leading to overuse of antibiotics which is a driving risk factor for AMR prevalence (Castro-Sánchez et al., 2016).

When each racial category was isolated and compared to the remainder of the population, there was only a significant relationship between AMR prevalence and percent of Non-Hispanic Asian in a population. Inappropriate use of antibiotics is well-known across Southeast Asia, particularly

in low- and middle-income countries (Holloway et al., 2017). In most countries, there are severe under-resourced communities, with financial and human resources preventing progress toward successful antibiotic stewardship programs. Many people originating from Asian countries, or who have cultural ties which may increase the frequency of travel to Asia, may contribute to the use of antibiotics by Asian community members. However, there is limited research available regarding AMR prevalence and antibiotic use in Asian populations in the U.S., highlighting a need for a more diverse representation in research on racial disparities relating to risk factors for AMR prevalence.

3GC resistance, carbapenem-resistance, and FQ resistance in bacterial-culturing outcomes for *E. coli* and KEC species did not show significant relationships between antimicrobial resistance and increases in percentages of females. While not significant, observed increases in AMR prevalence outcomes for increases in female percent are consistent with previous studies that indicate greater use of antibiotics among females (Schröder et al., 2016). Further exploration is needed to expand on the given limitations of the scope of this risk factor.

Decreases in AMR prevalence

Across all AMR outcomes, there were significant relationships between increasing concentrations of antibiotic-resistant bacteria and a s percentage aged 75 and over in the population. This data is not consistent with previous research which has indicated that older adults utilize a higher proportion of healthcare resources, including antibiotics, when compared to other age groups. Antibiotic prescription rates are also higher among those who are 75 and over, compared to lower age brackets (65 years to 74 years) (Kabbani et al., 2018), which we

would expect to relate to increasing AMR prevalence. This identifies a specific area of research that would be of interest for future research.

Although not significant, a decrease in antimicrobial resistance prevalence was seen with increases in percentage of uninsured and poverty. Uninsured status limits access to healthcare resources which may limit the access that individuals may have to antibiotic treatments. Insurance coverage and low-cost-sharing are important for access to medications including antibiotics. Poverty is another proxy variable for access to healthcare, and it is expected that poverty follows similar relationship trends as percentage uninsured. The opportunity for larger populations to contribute to the increase in antibiotic overuse and misuse and lead to increases in AMR prevalence could be less feasible for those living in poverty or are without insurance (Ungar & Ariely, 2005). While these relationships were not significant, it can be noted that significance measures were severely limited by the small sample size (n = 7). Therefore, definitive conclusions cannot be made regarding these variables but do warrant further investigation.

Other considerations for racial distribution across diverse AMR prevalence

Although there were no other significant relationships for racial distribution except for NH Asians, the models showed increases in AMR prevalence with increases in percentages of Hispanic and Non-Hispanic White populations and showed decreases in AMR prevalence with increases in percentage of Non-Hispanic Black. Much of the available literature on racial distribution of antibiotic prescriptions as the proxy for antimicrobial prevalence have focused on the Non-Hispanic White population. The observed increase would be consistent with previous

literature showing significant increases in antibiotic prescription fill rates in Non-Hispanic White individuals compared to non-White individuals. While Hispanic children have been previously prescribed less antibiotics compared to Non-Hispanic White (Goyal et al., 2017), trends of significantly higher non-prescription antibiotic use in Hispanic populations compared to Non-Hispanic may be contribute to the observed increase of AMR prevalence with increases in Hispanics (*Knowledge and Attitudes Regarding Antibiotic Use Among Adult Consumers, Adult Hispanic Consumers, and Health Care Providers — United States, 2012–2013*, n.d.).

There are gaps in the literature studying all-age Non-Hispanic Black relationships with AMR prevalence. While there is a limit to any analysis that can be done without a statistically significant relationship, the observed increase does align with previous research that has found that Non-Hispanic Black children are less likely to receive antibiotics compared to Non-Hispanic White children in the U.S. with identical pathogen diagnoses (Goyal et al., 2017). These trends could be influenced by racial bias from medical and healthcare providers (Hoffman et al., 2016). While using prescription rates as a proxy for AMR prevalence has limitations, consistent findings across these two measures support the transition to direct surveillance of quantified AMR prevalence and related risk factors.

Significance considerations

There was no statistically significant relationship between most of the variables tested, and it's important to note that many had a large standard error for their multivariable estimates in particular. The lack of significance in these relationships could be due to the small sample size (n=7) of the study or a true lack of a relationship between the variables. If there was a

relationship, it could be difficult to observe in this dataset. Increasing the sample size of the study would be expected to improve the validity of the results, to give a more statistically valid statement regarding the significance of these relationships.

Limitations

Sample Size: Due to the aggregation of census tract data to match the community level of the wastewater treatment plant outcome data, the resulting data set was very small (n=7). This introduced concerns with the scope of the conclusions that can be drawn from the analysis generated from a small sample size. While each sample is representative of a greater community, a cross-sectional study with a sample size this small can present interpretative challenges.

Data Cleaning Methods: Aggregating the Census demographic data with the methods included here did not account for the real-life distribution of residence within each census tract. This could introduce inaccuracies to our analysis if there is a significantly unequal distribution of households within the census tracts associated with the catchment areas for the WWTPs.

Colony Identification: The Blue colonies grown on 3GC-resistant and carbapenem-resistant media can be one of three bacteria; therefore, there is only so much you can read from the data. At the time, PCR was being used to confirm the specificity of the colonies being selected for *Klebsiella.*

Next Steps

Sample Size & Study Design - To improve the validity of the analysis and building from the correlations from this cross-sectional study, it would be beneficial to conduct a similar analysis in a longitudinal study which encompasses a larger number of wastewater plants or more

samples from these same sites over time (and thus a larger sample size). This could remain within the diverse north Georgia area or could branch out in collaboration with researchers from several cities to collect samples from other diverse regions of the United States.

Membrane Filtration - While this method was used to process the samples in this cross-sectional analysis, there have been studies done using bacterial culturing methods for wastewater samples which have not utilized membrane filtration, and have shown quantifiable results, indicating that it may be more efficient to leave this step out in the (Costanzo et al., 2005).

Spatial Regression - Given the geospatial element of community-level AMR wastewater surveillance, using spatial regression to map out different census tract variables with AMR prevalence could provide a better understanding of the spatial patterns of AMR transmission across neighborhood-level sewershed catchments. Successful use of spatial analysis in wastewater detection of pathogens was seen during the pandemic with analysis of SARS-CoV-2 wastewater detection in the Reno-Sparks metropolitan area in 2020 (Haak et al., 2022). Using a temporal sampling method with spatial analysis could help identify any statistically significant spatial trend, seasonal, and/or cyclic time series patterns of AMR prevalence (*Seasonal Pattern - an Overview | ScienceDirect Topics*, n.d.).

Additional AMR Detection Methods - Genotypic methods of AMR quantification are currently in the process of being developed as described in the methods section of this thesis, but results are not currently available from that analysis. PCR shows promise for systems with ready access to a laboratory setting and provides sensitive, targeted data for specific AMR gene surveillance. The project in which this thesis has been nested will continue to develop a PCR protocol for the detection of AMR genes in the Atlanta Fulton-Dekalb metropolitan area.

ddPCR - There are many other types of quantification methods that could be explored for processing environmental samples for AMR detection. Digital PCR (ddPCR) is a highly sensitive microfluidic PCR method, which has shown promise for the detection of AMR diagnostically (Wu et al., 2022) and in a variety of environmental samples (Carelli et al., 2022) as well as for other pathogens specifically detected in wastewater treatment samples (e.g., SARS-CoV-2). With the growing use of ddPCR for pathogen detection, especially with areas that may see both high and low prevalence of certain AMR target genes. To move toward the common use of ddPCR for AMR detection, there needs to be additional research conducted to determine ideal targets and for assay development that is specific to wastewater-level samples (Gonzalez et al., 2022).

PUBLIC HEALTH SIGNIFICANCE

The growing prevalence of antimicrobial resistance globally has serious clinical, financial, and environmental implications affecting human health. This affects both prevention and treatment efforts for persistent bacterial infections (Dadgostar, 2019). The increasing financial burden of AMR infections and treatments, mortality and morbidity rates associated with AMR, and the impacts of AMR environmental contamination can no longer be ignored. As we move toward a post-antibiotic era where common infections will no longer be treatable with antibiotics, surveillance and analysis of AMR patterns in diverse populations are crucial to support effective community-level intervention strategies.

While there have been recent efforts to quantify a variety of types of AMR prevalence, the relationships between AMR prevalence and social determinants of health (SDOH) remain severely understudied. While the small sample size of this study (n = 7) limits the real-world applicability of the findings, the relationships described can be used as a stepping stone toward further understanding the association between SDOH and AMR prevalence. Quantification of AMR prevalence in environmental samples may provide a more accurate picture of AMR transmission. Population-level analyses when paired with existing population-level SDOH data can help inform strategic preventative interventions better than clinical monitoring given the bias that exists in individual case monitoring systems. Developing cost-effective, sensitive, and specific protocols for environmental surveillance of AMR can help increase the accessibility of detection systems to other areas of the U.S. and across the globe. With more comprehensive and unbiased surveillance data, communities with the greatest need can be identified for targeted resource allocation and geographic-specific interventions in hopes to decrease AMR prevalence

and alleviate the associated burdens that come with high AMR prevalence more efficiently. Targeted resource allocation could ease the financial burden of AMR treatment and patient identification efforts and allow for greater allocation of funding towards the development of the next generation of antimicrobial treatments.

ACKNOWLEDGEMENTS

I would like to acknowledge and give thanks to my advisor, Dr. Marlene Wolfe, who made this work possible. Her guidance carried me through the various stages of writing this thesis and ongoing data collection for the greater project this thesis is nested within. I would also like to thank Dr. Maya Nadimpalli, the PI of the AMR in wastewater project, and the rest of the team that led the bacterial culture work, made up of Oluwatosin Olojo and Dania Hussain. They led the development of the bacterial culturing protocols and generously provided this data for this thesis. Thank you to Stephen Mugel for providing the census tract assignments, and to the Atlanta Department of Watershed Management and Fulton County Department of Watershed management for their participation in wastewater monitoring projects with Emory University. I would also like to thank my lab manager at the Center for Global Safe WASH, Orlando Sablon III, who provided continuous support and technical advice at multiple stages of this thesis development. Lastly, I would like to thank my parents, Ali and Shirley Sheikhzadeh, and Nicole Brynette Lewis for their continued support of my education and development as a researcher.

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