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Signature:

_____ _____

Andrew Rainey Date

Biogeographical Analysis of Antibiotic Resistance Genes in United States Wastewater

By

Andrew Rainey

Master of Public Health

Department of Environmental Health

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Karen Levy

Committee Chair

_____

Amy Kirby

Committee Member

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By

Andrew Rainey

B.S, University of South Florida, 2016

Emory University

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Thesis Committee Chair: Karen Levy, PhD MPH

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Abstract

Biogeographical Analysis of Antibiotic Resistant Genes in United States Wastewater

By Andrew Rainey

Antibiotic resistance is one of the biggest public health challenges of our time; accounting for about 2 million infected individuals and about 23,000 deaths from AR infections, costing about $55 billion annually in the United States. The purpose of this study is to provide data on the biogeographical distribution and concentrations of antibiotic resistance genes in United States influent wastewater samples. This project is a descriptive, hypothesis-driven study that will provide data from across the country that will help improve water quality, waste management and public health applications. Quantitative PCR assays of 10 clinically significant antibiotic resistant genes were conducted on influent wastewater samples that were collected from 49 wastewater treatment plants throughout the United States, chosen to represent influent wastewater from urban and rural areas. Urban samples had greater concentrations than rural for all targets except for *sul1*, *KPC* had the largest urban v. rural difference of 1.3074 Log10 copies/ml (p-value < 0.05). The difference of rural *sul1* to urban *sul1* levels was 3.2390 Log10 copies/ml (p-value < .0001). Regional differences were seen among the targets *NDM*, *OXA-48*, *sul1* and *tetW*, and the Mountain and West regions were most frequently different among these targets. The *mcr-1* gene is present in more states than clinical surveillance has discovered in the U.S. Urban areas are a major source of environmental ARGs and are more likely to provide positive samples. Clinically relevant ARGs are present at high quantities in U.S. wastewater and suggest urban areas and Mountain and Western states as major areas of interest.

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1.Literature Review

1.1 Antibiotic Resistance History

Bacteria are constantly interacting with each other, competing for space and nutrients to persist and reproduce [1-3]. One way to overcome opposing bacteria is to produce compounds to deter and destroy other organisms [1-6]. These compounds are antibiotics, which are described as molecules that inhibit or kill microbes [1, 3, 6, 7]. In order to defend themselves from these compounds, bacteria have developed mechanisms to overcome the effects from antibiotics [1-3, 6, 7]. Antibiotic resistance (AR) refers to bacterial mechanisms used to overcome or reduce the effects of an antibiotic [1, 7, 8]. It is natural phenomenon for bacteria to produce antibiotics and also to develop AR mechanisms [3, 9]. It was Rene Dubos in 1939 who was able to isolate bacillus species from the soil and show its bactericidal effects on gram-positive bacteria, being the first to show the natural production and application of antibiotics from bacteria in the environment [10, 11]. Paul Ehrlich developed the idea that there were compounds that could act as a “magic bullet”, targeting disease-specific organisms and not the host [2]. Alexander Fleming in 1929 was the first to discover this “magic bullet”, the natural antibiotic compound penicillin, and its potent action on common pathogenic bacteria [12, 13]. It wasn’t until 1940 that Ernst Chain and Howard Florey were able to purify penicillin and start proving its clinical significance in treating bacterial infections in humans [13, 14]. In this same year Chain was also able to show that there was enzymatic inhibition exhibited by some bacteria on penicillin, thus becoming the first to describe antibiotic resistance among pathogenic bacteria [13, 15]. After these discoveries the next 20 years became what is known as the golden age of antibiotic discovery [2, 6, 16]. More

than half of our antibiotics were discovered in this time period [6, 16, 17]. There are now over 100 antibiotics which are a part of 16 classes of antibiotics [13, 17]. Unfortunately, resistance has been described to almost every antibiotic used to date [7, 8, 16]. There is now a threat to human health due to a lack of newly discovered antibiotics and increased prevalence of resistance to existing antibiotics [7, 8, 16].

1.2 Antibiotic Resistant Gene Background and Mechanisms

β-lactams are a class of antibiotics that are the most widely used in the world today [2, 3, 18, 19]. β-lactams contain multiple groups of antibiotics including penicillins, cephalosporins, monobactams and carbapenems, all of which are classified based on its structure and activity [18, 20, 21]. β-lactam antibiotics all have a common β-lactam ring in its structure [18, 22]. β-lactams are also the oldest known antibiotic class to humans with its first discovery from Alexander Fleming and his discovery of penicillin in 1929 [2, 12, 18]. The process by which β-lactams are able to destroy bacteria is through its binding to specific enzymes, Penicillin-Binding Proteins (PBP) [1, 18]. Binding to PBPs interrupts peptidoglycan cross linking, thus inhibiting cell-wall formation [1, 18]. The first recorded observation of AR was to the β-lactam penicillin [15, 18]. β-lactams are prescribed based on their classes and the spectrum of targets that can be treated by the antibiotics [23, 24]. Penicillins are often the first choice to be used, then 1st, 2nd and 3rd generation cephalosporins and carbapenems are considered the last line of defense for β-lactam [23, 24]. Penicillins are not typically effective against gram-negative bacteria and 1st cephalosporins have shown to be more effective at treating these infections with less resistance displayed [23, 24]. When resistance to 1st gen cephalosporins were observed 2nd gen cephalosporins are used due to its increased coverage against β-lactamases [23].

The 3rd gen cephalosporins often have a broader spectrum and can be more effective against other gram-negative bacteria but resistant bacteria to 3rd gen cephalosporins are then considered to have extended-spectrum β-lactamases (ESBL) [23, 24]. Carbapenem antibiotics are considered a last resort treatment option, having the broadest spectrum against resistant bacteria [20, 21, 23, 24]. Bacteria that are resistant to all antibiotics including the carbapenems are considered carbapenems [20, 21, 23, 24] β-lactamases are enzymes that confer resistance to β-lactam antibiotics [18, 19, 25]. There have been more than 1,000 β-lactamases reported to date, and more are expected to be emerging [1]. β-lactamases are divided into 4 classes (A, B, C, D), based on its amino acid sequence and functional activity [1, 25]. β-lactamases are able to provide resistance to β-lactam antibiotics by hydrolyzing the chemical structures containing β-lactam rings [1, 19].

The class A β-lactamase *blaTEM* enzyme was first isolated in 1963 from *E. coli* that was isolated from a blood culture in Greece [26, 27]. This enzyme was the first plasma mediated serine active enzyme to be discovered in gram-negative bacteria [27, 28]. *blaTEM* has been known to be able to provide resistance against penicillins and narrow-spectrum cephalosporins, and in rare cases even against extended spectrum cephalosporins [27]. The *blaTEM* enzyme mechanism for AR occurs in two steps: acylation and deacylation [29]. The acylation step involves the removal of a proton and an attack from an oxygen to break the amide bond of the antibiotic to form an acyl-intermediate [29]. A water molecule is then used to break a covalent bond in the intermediate formation, leading to hydrolysis of the antibiotic [29]. This is mediated by a specific group of amino acids found on an omega loop located on the active site of the *blaTEM* enzyme [29].

The class A β-lactamase *Klebsiella pneumoniae* carbapenemase (*KPC*) enzyme was first isolated in 1996 from a clinical isolate of *K. pneumoniae* in North Carolina, United States [30]. *KPC* is a serine active plasma encoded enzyme [19, 31]. This enzyme is associated with gram-negative bacteria and has shown resistance to all classes of beta-lactam antibiotics and is thus considered a carbapenemase [20, 21, 31]. There have been 22 *KPC* variants described to date [20, 21]. The *KPC* enzyme has an active site that is similar to most beta-lactamases [20]. The most notable difference is that *KPC* has a smaller hydrophilic site and an active serine site at a shallower position, suggesting that it allows for easier access to carbapenems [20].

The class B β-lactamase New Delhi metallo-ß-lactamase (*NDM*) enzyme was first isolated in 2009 from a clinical isolate of *K. pneumoniae* in a Swedish patient that traveled to India [32]. *NDM* is a plasma mediated enzyme that has been found to be resistant to a large spectrum of beta-lactams, including almost every carbapenem antibiotic [32, 33]. Carbapenem antibiotics are often referred to as an antibiotic that is part of "the last line of defense" and resistance to this antibiotic is associated with severe, life-threatening infections [7, 33]. *NDM* is a Metallo-beta-Lactamase (MBL), meaning it shares a "distinctive αβ/βα sandwich fold of the metallo-hydrolase/oxidoreductase superfamily" [33, 34]. The *NDM* enzyme contains a shallow site in active region that contains two zinc ions that are flanked by two hairpin loops [33-35]. The loops are hydrophobic regions that aid in binding and catalyzing the reaction with the antibiotic, and when this is happening the hairpin loop is closed over active site with the zinc ions [33-35]. This catalyzed reaction opens the β-lactam ring, creating new complexes in the β-lactam where the zinc ions can bind [34, 35]. The first zinc ion binds to an open

carbonyl group and the second zinc ion binds an open nitrogen [34, 35]. After the zinc binding a tetrahedral adduct is formed and leads to cleavage of the β-lactam C-N bond, destroying the antibiotic [34, 35].

The class C β-lactamase *CMY* enzyme was first isolated in 1989 from a clinical isolate of *K. pneumoniae* in a wound patient in Seoul, South Korea [36]. This enzyme has mostly been found on the chromosome but has more recently been described to be plasmid-mediated, conferring resistance to cephalosporins, oxyimino cephalosporins, and aztreonam [28, 37]. The plasma-mediated *blaCMY-2* enzyme has the largest geographic distribution of all AmpC β-lactamases known [28]. AmpC enzymes are the most clinically relevant of all class C β-lactamases [1, 28]. Expression of AmpC is regulated through a transcriptional regulator, AmpR [1, 28]. AmpR remains bound to peptidoglycans normally, but when a β-lactam is present and starts to act on the cell wall there is an increase in peptidoglycan byproducts, creating a signal that results in the release on AmpR and the transcription of AmpC [1, 28]. Another mode of AmpC production also occurs through a mutation that leads to an increase in production an amidase, AmpD [1, 28]. AmpD production follows a pathway that leads to overexpression of AmpC [1, 28]. The AmpC active site is at the center of the molecule, splitting the two important regions, R1 and R2 [28, 38]. These two sites work together in the hydrolysis of the β-lactams [28, 38]. Catalytic residues in the AmpC enzyme include Ser64, Lys67, Tyr150, Asn152, Lys315, and Ala318 [28, 38].

The class D β-lactamase *OXA-48* was first discovered in 1965 and is an enzymatic group that is carbapenem resistant, but has also showed resistance to multiple other beta-lactams [19, 27, 39, 40]. The *OXA-48* gene was first identified from a clinical isolate of

K. pneumoniae that originated from Istanbul, Turkey [39]. There have since been many variants that are similar to *OXA-48,* all of which are determined to be different based on the nucleotide sequences [41]. The difference in the *OXA-48* β-lactamase that allows for it to provide carbapenem resistance is thought to be due to amino acid differences in its active site and in the β5-β6 loop of the structure of the enzyme [22]. This β5-β6 loop change in the structure provides a hydrophilic region of the enzyme to allow for greater binding to water, which is needed for hydrolysis of the antibiotic [22]. The *OXA-48* enzyme also has a large hydrophobic region that helps ensure that the antibiotic is pushed towards the hydrophilic end that leads to hydrolysis [22].

Methicillin is a semi-synthetic penicillin that was originally developed as a response to the development of resistance to penicillin [42, 43]. Resistance to methicillin was almost immediately seen in *Staphylococcus aureus* infections in Europe [42, 44]. The *mecA* gene which confers methicillin resistance was first discovered in *S. aureus* bacteria and the gene was determined to be carried on a mobile genetic element called a cassette chromosome (SCCmec) [45-47]. *MecA* production is regulated through a signal transduction system associated with two regulatory genes (*mecR1 and mecI*) which located next to the *mecA* gene in the cassette, creating a mec gene complex [48]. MecR1 is an integral-membrane zinc dependent sensor and MecI is a transcriptional repressor [48]. Binding of the antibiotic to the penicillin-binding domain of MecR1 activates the intracellular metalloproteinase domain of MecR1, leading to a process that is believed to then lead to degradation MecI, allowing for transcription of the *mecA* gene [48]. The *mecA* gene encodes for the penicillin-binding protein (PBP2a) [48]. This is the protein that is targeted by β-lactams that leads to a disruption in cell wall synthesis [1, 18, 48].

The PBP2a protein that is produced from *mecA* inhibits binding of the antibiotic, allowing for cell wall synthesis of the bacteria to continue as usual [48, 49].

Glycopeptide antibiotics are a class that date back to the 1950's with the first discovery in the class coming from Eli Lilly, discovering vancomycin from soil samples taken from a jungle in Borneo [50, 51]. Vancomycin resistance was discovered almost 30 years later in samples of enterococci (VRE) [52, 53]. A few years after vancomycin resistance was identified the *vanA* gene was discovered [54, 55]. The *vanA* gene has been identified as a chromosomal mutation and has also been found on plasmids [56, 57]. Vancomycin's action on bacteria is through inhibition of cell wall synthesis [56]. The *vanA* gene encodes for a d-Ala d-Lac ligase which follows a pathway that creates a peptidoglycan precursor that does not allow for cell wall synthesis to continue as usual [56, 58].

Polymyxins are a group of antibiotics that have been recognized as being effective against many gram-negative bacteria with little resistance [59]. Colistin is a "last resort" polymyxin that is often used to treat infections of gram-negative bacteria that are resistant to all other administered drugs [60]. Colistin is often considered for use after all else has failed due to its toxic effect on the treated patients, causing colistin induced nephrotoxicity and neurotoxicity [59, 61, 62]. Colistin resistance was first believed to be induced exclusively through chromosomal mutations, but in 2015 the first colistin resistant plasmid-mediated gene was discovered in China, *mcr-1* [63]. There have been additional discoveries of *mcr* genes such as *mcr-2* which was discovered in Belgium, *mcr-3* which was discovered in China, *mcr-4* first showing up in Italy from a pig slaughter sample from 2013, *mcr-5* discovered simultaneously from pigs in China and

from an isolate of *Salmonella paratyphi B* in Germany and ICR-Mo which is an intrinsic *mcr-1*-like homolog from a *Moraxella osloensis* specimen [64-69]. Colistin targets the cell by binding with the lipopolysaccharide of the outer membrane of the bacteria [70, 71]. Colistin is attracted to negatively charged phosphate headgroups of lipid A, leading to disruption and eventual lysis of the cell [70, 71]. *Mcr-1* codes for a "phosphoethanolamine (PEA)-lipid A transferase that adds a PEA group to the 1(4')-phosphate of glucosamine moieties in LPS-lipid A of the bacterial outer membrane via a putative ping-pong mechanism, dampening the net negative charge and consequently reduces the affinity of colistin" [71, 72].

Sulfonamides are the oldest class synthetic antibiotics used by humans dating back to the 1930's, all of which contain the same functional group in its molecular structure [57, 73]. Sulfonamide antibiotics are able to target a wide range of bacteria, both gram-positive and gram-negative [57, 74]. Resistance was almost immediately reported after the introduction of sulfonamides [3, 57]. There are now four recognized sulfonamide resistant genes, with the latest one being discovered in 2017 (*sul4*) [75]. The first sulfonamide gene to be discovered was *sul1*, being discovered in *E. coli* in 1983 in Sweden [76]. The first three sul genes that were discovered (*sul1-3*) have all be found to be plasma-mediated, while the *sul4* gene was found in a gene cassette on a class 1 integron [57, 75]. Sulfonamides target the enzyme dihydropteroate synthase (DHPS), which is involved in the folate pathway and it inhibits the formation of dihydrofolic acid, destroying the bacteria [74]. *Sul1* provides resistance to sulfonamides by coding for an alternative DHPS enzyme that has a low affinity for the antibiotic [74].

The tetracycline class of antibiotics were first discovered in 1948 by Benjamin Duggar in the United States from the bacterium *Streptomyces aureofaciens*, which was found in soil samples [77]. Tetracycline antibiotics are considered to be "broad spectrum" due to their ability to treat a vast array of bacterial infections [57, 78]. Tetracycline resistance was first seen in 1953 from the bacterium *Shigella dysenteriae* [57, 78, 79]. There have been over 40 different tetracycline resistance genes discovered [57]. Tetracycline resistance genes are determined to be new if they share less than 79% of their amino acid sequence with all other known *tet* genes [57]. The *tetW* gene was first discovered in 1999 from obligate anaerobic bacteria collected from the rumens of animals [80]. The *tetW* gene is a chromosomal gene, located on a transposon, and has been found in gram-positive and gram-negative bacteria [57, 81]. The *tetW* gene has the third largest host range among all tetracycline resistance genes [81]. Tetracycline enters the bacterial cell through pore channels, following a pathway that eventually inhibits protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor site, halting protein elongation [57, 78, 82]. Resistance to tetracyclines occur through three different mechanisms: energy-dependent efflux pumps, ribosomal protection proteins (RPPs), and enzymatic inactivation [57, 78, 82]. The *tetW* gene uses RPPs, encoding for a protein that will act by releasing tetracycline from the ribosome, allowing for the normal process of protein synthesis to occur in the bacteria [78, 81, 82].

1.3 Mechanisms of Antibiotic Resistance Development

AR can be developed in bacteria through multiple mechanisms [83, 84]. The first is through natural mutations in the bacterial genes [84-86]. This natural process does not require any external stimulus [84-86]. Mutations and evolution of bacterial genes occur at

variable rates but can be very high [84, 86]. Mutation rates are mediated through a vast amount of factors, some of which can be beneficial, and some can lead to destruction of the bacteria [84, 86]. Natural selection will allow for those certain bacteria carrying beneficial mutations to survive and reproduce when other bacteria are killed by the antibiotic [84, 86]. The other mechanism is horizontal gene transfer (HGT) which includes: conjugation, transformation and transduction [83]. Conjugation is a process in which DNA, specifically plasmid DNA, is transferred through direct cell-to-cell contact [83]. Transformation is a process in which bacteria take up exogenous DNA from the natural environment [83]. Transduction is a process in which DNA is inserted into bacterial cells from bacteriophages [83].

ARGs can be found in bacterial chromosomes or extrachromosomal DNA like plasmids. Transposons and integrons are forms of mobile DNA that can be found within bacterial chromosomes and plasmids [85, 87-90]. Integrons are mobile genetic elements capable of excision and reinsertion into segments of DNA [85, 87, 88, 90]. Integrons are sites of genetic aggregation and are able to insert genetic sequences and introduce reassortment of the sequences in the DNA [85, 87, 88, 90]. Integrons are often associated with carrying ARGs [85, 87, 88, 90]. ARGs in integrons can be aggregated in specific short sequence regions called gene cassettes [85, 87, 88, 90]. Gene cassettes can be integrated into DNA through the enzyme integrase, which is coded by the integron [85, 87, 88, 90]. Transposons are genetic elements, which often carry ARGs, can successfully move to different locations within the chromosomal DNA and also jump to extrachromosomal DNA and back [85, 89, 90]. The movement of transposons within chromosomal DNA often causes mutations in the DNA leading to the expression of the

ARGs [85, 89, 90]. The ability of transposons to move into plasmids means that it can integrate itself with ARGs into the plasmid which can then be transferred to other bacteria [85, 89-92]. Transposons can also be integrated into bacteriophage DNA [85, 89-92]. A bacteriophage is a bacterial virus. Transposons carrying ARGs that integrate into bacteriophage DNA can then be transferred to other bacteria through transduction [85, 89-92].

1.4 United States Policy on Antibiotic Resistance

AR is one of the greatest public health threats of our time and is a major worldwide threat to global health, food security and development [8]. AR has been found is every region of the world and the trend in global travel has made it easier for the antibiotic resistant bacteria (ARB) to be spread to people and animals throughout the world [93]. The AR threat has become so great that it now requires political action and commitment. The United States government has recognized the AR issue being both domestic and global and has started to take actions aimed at understanding and mitigating the threat. In 1999, the United States federal government created the Interagency Task Force on Antimicrobial Resistance (ITFAR) which "is a coordinating committee within the Federal Government for research on antimicrobial resistance"[94]. ITFAR is co-chaired by the Centers for Disease Control and Prevention (CDC), Food and Drug Administration (FDA), and National Institutes of Health (NIH), and also works with many more government agencies within the United States Federal Government [94]. In 2001, ITFAR released their initial plan entitled *A Public Health Action Plan to Combat Antimicrobial Resistance, Part I: Domestic Issues* [95]. This plan outlines goals and actions to complete each goal, all of which are aimed at addressing the issue of AR [94]. The goals laid out

from ITFAR fall under four focus areas: surveillance, prevention and control, research, and product development [94]. The ITFAR plan was focused mainly on clinical AR and hospital acquired infections (HAI), and it was also focused on agriculture-derived AR [94]. Environmental AR did not fall under any of the goals in the plan. On September 18, 2014 President Barack Obama signed Executive Order 13676, Combating Antibiotic-Resistant Bacteria (CARB) [96]. This order affirmed that "the Federal Government will work domestically and internationally to detect, prevent, and control illness and death related to antibiotic-resistant infections by implementing measures that reduce the emergence and spread of antibiotic-resistant bacteria and help ensure the continued availability of effective therapeutics for the treatment of bacterial infections" [96]. The U.S. National Strategy for Combating Antibiotic-Resistant Bacteria was released alongside Executive Order 13676 and a report on combating antibiotic resistance by the President's Council of Advisors on Science and Technology (PCAST) [97, 98]. The U.S. National Strategy outlined five main goals to be used when guiding actions for the federal government: "1) Slow the emergence of resistant bacteria and prevent the spread of resistant infections, 2) Strengthen national One Health surveillance efforts to combat resistance, 3) Advance development and use of rapid and innovative diagnostic tests for identification and characterization of resistant bacteria, 4) Accelerate basic and applied research and development for new antibiotics, other therapeutics, and vaccines objectives and 5) Improve international collaboration and capacities for antibiotic resistance prevention, surveillance, control, and antibiotic research and development [97]. The PCAST report provided three main recommendations for tackling antibiotic resistant bacteria: "1) Improve tracking antibiotic-resistant bacteria, 2) Increase the life of current

antibiotics by improving use and implementing interventions and 3) Increase speed to discover and develop new antibiotics and other interventions" [98]. In March 2015, The U.S. National Action Plan for Combating Antibiotic-Resistant Bacteria (National Action Plan) was developed by the Interagency Task Force for Combating Antibiotic-Resistant Bacteria in response to Executive Order 13676 [99]. The National Action Plan lays out objectives that will help the U.S. achieve each of the five goals that were initially set in the National Strategy over five years (2015-2020) [99]. These objectives are accompanied with milestones set at one, three and five years in the future to describe what is expected out of each objective in the National Action Plan [99]. Objective 4.1 in the National Action Plan is to "Conduct research to enhance understanding of environmental factors that facilitate the development of antibiotic-resistance and the spread of resistance genes that are common to animals and humans" [99]. An annual milestone for this objective is to "ensure that U S Government research resources are focused on high-priority antibiotic resistance issues (including basic research on the emergence and spread of resistance genes)" [99]. Environmental AR would fall under this objective and should be a research area of importance for the U.S. Government. In October 2017 a progress report was released for the first two years of the National Action Plan [100]. This plan describes actions and progress made for each objective in all of the goals set by the National Action Plan [100]. The report recognizes progress in the research of antibiotic resistant bacteria and genes in our environment that is helping build data sets that can be used by government agencies and the public in the future [100]. However, there is still much to be gained in this field.

Antimicrobial pesticides fall under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), thus are regulated in a similar manner as other pesticides in the U.S., with a few slight differences in the data requirements for pesticides, and the development of a Use Site Index (USI) for antimicrobial pesticides [101, 102]. The Environmental Protection Agency (EPA) enforces FIFRA, which is "the Federal statute that governs the registration, distribution, sale, and use of pesticides in the United States" [103, 104]. "FIFRA enforcement is focused on the sale, distribution, and use (which can include disposal) of pesticides" [104]. The main purpose of FIFRA is to ensure a pesticide "will not generally cause unreasonable adverse effects on the environment, taking into account the economic, social, and environmental costs and benefits of the use of any pesticide" [103, 104]. "FIFRA defines the term "unreasonable adverse effects on the environment" to mean the following: any unreasonable risk to man or the environment, taking into account the economic, social, and environmental costs and benefits of the use of any pesticide, or any human dietary risk from residues that result from use of a pesticide in or on any food inconsistent with the standard under section 408 of the Federal Food, Drug, and Cosmetic Act (FFDCA)" [104, 105]. Section 408 of FFDCA authorizes EPA to set tolerances, or maximum residue limits, for pesticide residues on foods [106]. Data differences between antimicrobial pesticides and all other pesticides fall under subpart W of 40 CFR part 158, *Data Requirements For Pesticides* [107]. The FFDCA in an act that gave the FDA power to oversee the safety of food, drugs, medical devices and cosmetics [106]. This act deals with antimicrobials that are applied directly in or on food, or on any substances that will come into contact with food [106, 108]. Substances that can come into contact with food can be food packaging, food

packaging surfaces, or any other non-packaging food contact surfaces [106, 108]. The Food Quality Protection Act of 1996 (FQPA) amended both FIFRA and FFDCA by changing the definitions of what food additives and pesticide chemicals are [108, 109]. A major change from this act was by switching the regulatory authority from EPA from FDA regarding some food contact antimicrobials that were originally considered food additives by FDA, to now being considered pesticide chemicals by EPA [108, 109]. The Antimicrobial Regulation Technical Corrections Act of 1998 (ARTCA) was another act that amended the definition of a pesticide chemical [108, 110]. The transfer of authority from FDA to EPA on those select food contact products, as defined from FQPA, was unintended and ARTCA corrected those unintended transfers back to EPA [108]. ARTCA also allowed some regulatory authority on raw agricultural commodities by FDA, while the majority was still under the regulatory authority of EPA [108].

Currently there are three antibiotic pesticides used for control of plant diseases in the United States: Streptomycin, Oxytetracycline and Kasugamycin [111]. Tolerance level for residuals of the antibiotics are set for each approved food product that is treated with the antibiotics. The tolerance levels are set under 40 CFR part 180.245 of FIFRA [103]. Streptomycin is used in various crops such as celery and potatoes and has tolerance levels that vary with each crop it is used for [103, 112]. In March, 2017, Streptomycin was approved for use on citrus under an emergency exemption that set the residual tolerance to 2 parts per million (ppm) for fruit and 6 ppm for dried pulp [113]. Oxytetracycline was originally used for apples with a residual tolerance max at 0.35 ppm [103, 114]. In March, 2017 Oxytetracycline was also approved for use on citrus under emergency exemptions with a residual tolerance set to 0.40 ppm [115]. As of December 4, 2018,

Oxytetracycline was officially allowed to be used on citrus with a tolerance level of 0.01 ppm [116]. The time-limited emergency exemption tolerances set for streptomycin and oxytetracycline will expire December 31, 2019[113, 116]. Under section 18 of FIFRA, EPA is allowed to set these emergency tolerances without providing notice or period for public comment [103, 104]. Section 408(b)(2)(A)(i) of FFDCA allows EPA to establish these tolerances only if EPA determines they are "safe", which is defined here to mean that "there is a reasonable certainty that no harm will result from aggregate exposure to the pesticide chemical residue, including all anticipated dietary exposures and all other exposures for which there is reliable information" [106]. The Florida Department of Agriculture and Consumer Services (FDACS) gained approval under this process for the use of Streptomycin and Oxytetracycline on citrus in Florida "to suppress the *Candidatus Liberibacter asiaticus* (C Las) bacterium that causes Huanglongbing (HLB) also known as citrus greening" [113, 115, 117]. Florida citrus production has seen an "overall decrease in production of more than 60% primarily due to HLB" thus requiring this emergency action of antimicrobial pesticide applications on citrus [113, 115, 117]. Kasugamycin is currently used on walnuts, vegetables, fruits and cherries, with no current emergency use and the tolerance levels range from 0.04-0.6 ppm [118]. There is a lack of human health studies on the long-term ingestion of crops with antibiotic residues [119, 120].

The FDA has developed guidelines in the Animal Drug Availability Act of 1996 (ADAA) regarding antibiotic use in animals, recognizing the link from agriculture to human health [121]. In 2015 the FDA implemented the veterinary feed directive (VFD) drugs section into the ADAA [122]. The FDA decided that a "VFD drug is intended for

use in animal feeds, and such use of the VFD drug is permitted only under the professional supervision of a licensed veterinarian [122]. The FDA have also released guidance for industry (GFI) #209 and 213 which are both recommendations on veterinary stewardship of antibiotics in agriculture, however this guidance are not legally binding [123, 124]. California became the first state to create law regarding antibiotic use with Senate Bill-27 Livestock: use of antimicrobial drugs [125]. The bill "prohibits the administration of medically important antimicrobial drugs, as defined, to livestock unless ordered by a licensed veterinarian through a prescription or veterinary feed directive pursuant to a veterinarian-client-patient relationship, as specified, and would prohibit the administration of a medically important antimicrobial drug to livestock solely for purposes of promoting weight gain or improving feed efficiency" [125]. Maryland has followed suit and has passed legislation in Senate Bill 422 Keep Antibiotics Effective Act of 2017 [126]. All other federal actions to combat AR come in the form of guides and recommendations that have mostly targeted antibiotic stewardship though environmental aspects have begun to be recognized for its important role in AR [127, 128]. In 2015 the White House Forum on Antibiotic Stewardship was "created an opportunity for more than 150 leaders in government, healthcare, agriculture, and industry (e.g., pharmacy) to discuss ways to improve antibiotic use and slow the spread of antibiotic-resistant germs" and these leaders all agreed to implement their changes over the next five years after the forum [129]. In 2018 the U.S. Government launched the AMR Challenge, which "is a way for governments, private industries, and non-governmental organizations worldwide to make formal commitments that further the progress against antimicrobial resistance" [128]. The AMR challenge asks for commitments in at least one of five specific areas:

tracking and data, infection prevention and control, antibiotic use, environment and sanitation and vaccines, therapeutics, and diagnostics [128]. The specific commitment area focused on environment and sanitation is aimed to “decrease antibiotics and resistance in the environment, including improving sanitation” [128].

There has been progress in our knowledge, but not much in terms of slowing down the growing AR problem in the United States. There is a need for more policy options to help mitigate the AR issue in the country. Future policy options should recognize the intricacies involved with antibiotic resistance development, transfer and dissemination among people, animals and the environment [130-133]. Before more policy can be created there is also a need for more research into risk assessment of ARB and ARGs to identify and characterize factors such as the greatest risk areas, sources, pathways, drivers, key points of exposure, points of control and treatment options [119, 120, 130-135]. Once more research into the basic science involving ARB and ARGs is completed and we have a better understanding of the ecology of the situation, effective policy options can start to be formulated and implemented [119, 131, 133, 135, 136]. Increased funding and basic research will provide us with the knowledge we need. More programs like the AMR challenge, funding research, is one way of obtaining the knowledge we need to create effective policy options. Policy options regarding ARB and ARGs should be developed as soon as possible in anticipation of the growing problem, rather than creating policy as a reaction to a problem that has already arrived [119, 136].

1.5 Antibiotic Resistance Burden and Statistics

Currently, the Centers for Disease Control and Prevention (CDC) estimate that about 2 million people are infected with ARB, and about 23,000 people die from AR infections

annually [7]. Many more deaths occur in people due to other medical complications that were exacerbated from AR infections [7]. There have been only a few estimations of the burden of AR globally and of those estimations it has been noted that they are both unreliable and not substantial enough to provide quality evidence on subject [7, 137, 138]. The most comprehensive global burden estimate of AR came from the *Review on Antimicrobial Resistance*, commissioned by the UK Prime Minister, publishing their final report in May 2016 [139]. This review estimated both health and economic burdens of AR. The review provides a low estimate that there are about 700,000 deaths caused by AR each year and that by 2050 this number could be as high as 10 million per year [139]. The review also estimated that this would lead to a 2-3.5% reduction of global Gross Domestic Product (GDP), resulting in a cumulative loss of about $100 trillion USD [139]. One domestic study estimated that the United States loses about $55 billion USD per year due to AR ($20 billion in health service costs and $35 billion in lost productivity) [140]. Another study in Chicago looking at high-risk adult patients in a hospital found that "medical costs attributable to antibiotic resistant infections (ARI) ranged from $18,588 to $29,069 per patient, excess duration of hospital stay was 6.4–12.7 days, attributable mortality was 6.5%, and the societal costs were $10.7–$15.0 million" [141]. These estimates are very limited and do not describe the entire situation surrounding the burden of AR. Accurate and precise U.S. estimates are not yet available and there are many limitations to obtaining better estimates at this time due to multiple reasons [7].

1.6 Surveillance of Antibiotic Resistance in the United States

The National Antibiotic Resistance Monitoring System (NARMS) is an interagency public health surveillance system that was created in 1996 and it monitors antibiotic resistance in enteric and foodborne bacteria [142]. NARMS is comprised of the CDC, FDA, United States Department of Agriculture (USDA) and state and local health departments [142-144]. The main goals of NARMS are to: "monitor trends in antimicrobial resistance among enteric bacteria from humans, retail meats, and animals, disseminate timely information on antimicrobial resistance in pathogenic and commensal organisms to stakeholders in the United States and abroad to promote interventions that reduce resistance among foodborne bacteria, conduct research to better understand the emergence, persistence, and spread of antimicrobial resistance and provide data that assist the FDA in making decisions related to the approval of safe and effective antimicrobial drugs for animals" [145]. Each agency looks for AR enteric bacteria from different sources. CDC monitors AR enteric bacteria from human clinical samples [146]. FDA monitors AR enteric bacteria in retail meat products [143]. USDA monitors AR enteric bacteria in food animals [144]. Each agency creates annual reports on the data that was collected, along with a comprehensive integrated report of all the data collected [147, 148]. NARMS has been useful in monitoring outbreaks, providing data on emerging AR issues and also providing data to help guide policy [145].

The Antibiotic Resistance Laboratory Network (ARLN) was established in 2016 and is maintained by the CDC, who provide support and help build lab capacity of state and local health laboratories in all 50 states and Puerto Rico [149]. There are seven regional labs in ARLN, who all help in coordinating and completing activities with the CDC [149]. ARLN also allows CDC to respond to reported local outbreaks and to work

with the health departments to respond appropriately [149]. The CDC also is able to make some isolates collected through the labs available for further investigation through the AR Isolate Bank [149, 150].

The USDA has several AR surveillance systems in addition to NARMS: the National Animal Health Monitoring System (NHAMS), National Animal Health Surveillance System (NAHSS) and National Animal Health Reporting System (NAHRS) [151-153]. These systems broadly monitor the health and management of domestic livestock and poultry in the United States, while giving specific attention to AR pathogens [145, 151-153]. Surveillance in the United States is constantly being pushed to be more responsive, expansive, proactive and vigilant while using cutting edge technologies [145].

There is still a need for environmental surveillance for AR drivers and sources. There is no national surveillance system looking at the waters in the United States for AR bacteria. A well-developed national environmental surveillance system could identify sources of contamination, selective pressures, hot spots for AR dissemination and exposure, or key points for mitigation to prevent dissemination or exposure to ARB.

1.7 Clinical Antibiotic Resistance

Antibiotics are seen as one of the most important discoveries in human history; regarded for its contributions leading to improved lifespans, reductions in diseases and deaths and an overall improvement in life [3, 7, 16, 57, 127]. However, the threat of resistance to antibiotics has been following close behind after each advance in the field ever since the initial discovery of antibiotics and its use in humans [7, 16]. AR is estimated to cause millions of illnesses and thousands of deaths in the United States each

year, however these estimates are believed to be an underestimation of the actual burden [7, 127]. Infections from ARB are a threat to every human [7, 127]. ARI that are seen in clinical settings mostly come from the community, but infections can also be acquired in the clinical settings and the development of these clinical ARI can mostly be attributed to the antibiotics we use [16, 154]. The effectiveness of the antibiotics we use on these infections are dwindling and options are running out [7]. There has been a major stall in the development of new antibiotics recently and this has led to clinicians using the same antibiotic over and over again [16, 154]. When a new antibiotic is introduced there is a period where it remains highly effective [16, 154]. The effective life of a new antibiotic can be extended through proper stewardship practices [154]. Antibiotic stewardship refers to the manner in which clinicians prescribe and use antibiotics in patients [127]. Improving antibiotic stewardship is believed to be the most important method to protect patients and combat antibiotic resistance [127]. The CDC estimates that each year 30% (~ 47 million) of all antibiotic prescriptions in the United States were unnecessary [127]. AR infections are estimated to add an additional 6.4 to 12.7 days in the hospital for a patient [16]. Improved stewardship would decrease the propagation of AR, decrease overall hospital and patient costs and improve patient outcomes [16, 127]. Judicious use of antibiotics helps ensure that they can continue to be effective in the future for all patients [16, 127]. Improved stewardship aims to make sure that clinicians will only use the correct antibiotics when necessary [16, 127]. Effective stewardship will make sure that each patient is provided with optimal care and timely treatment [16, 127].

Infections from gram-negative bacteria are the most urgent threats in our country and the most serious of these infections are healthcare associated [7]. Hospital acquired

infections (HAIs) are of high concern because the target population is likely to already be immunocompromised since they are being treated in a healthcare facility [127, 155, 156]. AR HAIs have been mainly driven by the misuse of antibiotics [127, 155]. The most common HAI comes from the bacterium *Clostridium difficile* [127, 155, 156]. *C. difficile* infections are the leading cause for gastroenteritis deaths in the United States, causing about 14,000 deaths each year [156]. Hospital acquired *C. difficile* infections have estimated to represent as much as 65% of the overall *C. difficile* infections in the United States [156]. Since the emergence of an AR strain of *C. difficile* in the year 2000 the CDC saw a 400% increase in deaths attributed to the infection from the years 2000-2007 in the United States [7].

Other sources of AR clinical infections are the community and the environment [157, 158]. The community can include factors such as human contact, occupation, the household, travel related factors or diet [157, 158]. ARB can be spread among populations through human contact and interactions [157, 158]. This human-human transfer of ARB can lead to infections and spread of the bacteria throughout different geographic areas [157, 158]. Global travel is an activity where millions of people carry ARB that are resistant to specific antibiotics that may only be used in regions outside of the United States [158]. People may be exposed to these foreign ARB while traveling abroad or the bacteria may be brought in from an individual traveling into the United States, ultimately lead to the possibility of establishment of these ARB in the country [158]. International travel is thought to be highly associated with ARI [158]. Occupations can also put people at risk of developing or transferring ARB [158, 159]. Workers in animal production have been seen to play a role in acquiring and transferring ARB [158,

159]. The use of antibiotics in agriculture have led to the emergence of many ARB in the food products produced from the animals [7, 159]. These contaminated food products can provide an opportunity of exposure to many workers including the farm workers, veterinarians, food processers, packers, and retail workers [159]. At any time these people can be exposed during their job to ARB or vice-versa, contaminating the meat products and making future exposure to those that eat the food [159]. ARGs can also be transferred to other pathogenic bacteria throughout this process [159]. All workers that handle these food products must always practice proper food handling to protect themselves and the consumer [159]. Another occupation that has been studied is pharmaceutical workers [160]. These workers can often be exposed to sub-inhibitory levels of antibiotics through inhalation, ingestion or dermal absorption throughout their workdays [160]. A study looked at workers in the field, comparing pharmaceutical workers and non-workers [160]. This study found that *Staphylococcus, Pseudomonas and E. coli* species from the pharmaceutical workers were more resistant and resistant to more drugs than people that did not work in the pharmaceutical field [160].

β-lactams are a class of antibiotics that are the most widely used in the world today [2, 3, 18, 19]. β-lactams contain multiple groups of antibiotics, the most common are penicillin, methicillin, cephalosporin and carbapenems, all of which are classified based on its structure and activity [18, 20, 21]. AR to β-lactams is seen in many gram-negative bacterial infections [7]. Extended-spectrum β-lactamase allows for bacteria to be resistant to a wide range of β-lactams such as penicillins and cephalosporins [7]. Penicillins are used commonly because of its ability to treat a broad range of infections, but are not often used to serious infections because resistance to this antibiotic is common in the United

States [7]. A common clinical infection resistant to penicillin is methicillin-resistant *Staphylococcus aureus* (MRSA) [7]. MRSA is estimated to cause 80,000 infections and 11,000 deaths annually in the United States [7]. MRSA is a term that is also used to describe any strain of *S. aureus* that is resistant to cephalosporin and not just methicillin [7]. MRSA resistance to cephalosporins has become a point of concern [7]. The most urgent cephalosporin resistant bacterial infection is caused by *Neisseria gonorrhoeae* [7]. There is an annual estimate of 820,000 *N. gonorrhoeae* infections, with 246,000 of those being resistant to any antibiotic and almost 5% (11,480) of those being resistant to cephalosporins [7]. AR infections cause by ESBL-producing Enterobacteria are a serious threat in the United States [7]. It is estimated that around 140,000 Enterobacteriaceae infections occur each year in the United States [7]. Enterobacteriaceae infections are most often cause by *Klebsiella spp.* and *E. coli* [7]. Of these cases, 26,000 infections and 1,700 deaths are believed to be caused by ESBL-producing Enterobacteria [7]. Carbapenem resistance is of high clinical importance because these drugs are used to treat serious infections [7, 18, 19]. Carbapenem use in hospitals from 2006 to 2012 increased by 37% [127]. Carbapenem resistant Enterobacteria infections are categorized as an urgent threat in the United States, estimated to cause 9,300 infections and 610 deaths annually [7].

Vancomycin use in hospitals from 2006 to 2012 increased by 32% [127]. Vancomycin is often the treatment option for MRSA since it remains effective and only 13 cases of vancomycin resistant *S. aureus* have been detected in the United States since 2002 [7]. Vancomycin resistant Enterococcus infections are considered a serious threat in the United States [7]. Enterococcus infections are often associated with hospital and other clinical settings [7]. Annually, it is estimated that there are 66,000 healthcare-associated

Enterococcus infections [7]. 20,000 of those infections occur in hospitalized patients and are vancomycin resistant, causing an estimated 1,300 deaths [7].

Colistin is a last resort drug that is only used in patients that have infections that are resistant to all other antibiotics [60]. Colistin resistance surveillance is of the upmost importance in the United States due to the serious health implications of colistin resistant bacteria [60]. To date the CDC has identified 53 human clinical and 2 animal isolates of colistin resistant bacteria in 21 states [60]. The most cases (16 human isolates) have come from Massachusetts [60].

Sulfonamides are often used as a first-line drug because of its ability to target a wide range of bacteria, both gram-positive and gram-negative [7, 57, 74]. Shigella infections resistant to these drugs are considered a serious threat in the United States [7]. Shigella is estimated to cause 500,000 infections and 40 deaths each year [7]. 27,000 of these infections are cause by AR Shigella species [7]. From 2007-2011 Shigella infections that are resistant to sulfonamides have increased from under 30% to almost 70% [7]

Tetracyclines are not usually first-line option treatments for gram-negative infections, some of which are used for multi-drug resistant infections [7]. The most urgent tetracycline resistant bacterial infection is caused by *Neisseria gonorrhoeae* [7]. There is an annual estimate of 820,000 *N. gonorrhoeae* infections, with 246,000 of those being resistant to any antibiotic and 23% (188,600) of those being resistant to tetracyclines [7].

1.8 Environmental Antibiotic Resistance

The environment is the major reservoir of ARB and ARGs [119, 134, 135, 161-163]. The environment is where ARGs are disseminated and transferred to other bacteria

[134, 135, 161, 162]. The connection between ARGs and ARB in the environment and clinical outcomes is not yet well researched [135, 157, 161-163]. Regardless, the risk is present and suspected to be increasing [157, 161-164]. Current research questions are focused on a few different aspects regarding the clinical impact and burden from environmental ARGs and ARB [5, 134, 157, 161-163]. There is a need to quantify the sources of clinical AR cases to determine the overall contribution from the environment [119, 134, 135, 157, 162, 163]. The transfer rates of ARGs to other potentially pathogenic bacteria in humans is not yet well understood [134, 135, 157, 163]. There is also a gap in knowing how often exposure to ARGs in non-pathogenic bacteria can lead to transfer of those genes to other bacteria in humans, leading to potential AR infections [119, 134, 135, 157, 162, 163]. There is also an interest in knowing ARG transfer to colonizing organisms in humans from environmental bacteria [135]. Colonizing bacteria harboring ARGs could pose a risk to transfer of those genes to other bacteria, leading to adverse clinical outcomes [135]. Dominant pathways and pathogens harboring ARGs in the environment also have yet to be clearly defined to identify the greatest environmental risks to ARG exposure [119, 134, 135, 157, 162, 163]. Finally, it has yet to be determined which genes are most prevalent in the environment and which are more likely to make it into the clinical setting [119, 134, 135, 157, 162, 163]. Answering these questions will help solidify the quantitative and qualitative factors that contribute to clinical AR infections.

The major environmental reservoirs for ARGs are the soil and water [5, 119, 134, 161, 164-166]. ARGs can enter the environment from many sources and cycle throughout multiple compartments in the environment [5, 119, 134, 161, 164-166]. The major source

of ARGs entering the environment is through wastewater containing human and animal feces [5, 119, 134, 161, 164-166]. Wastewater containing human and animal feces contain large amounts of bacteria, some of which contain ARGs [5, 134, 161, 164, 166]. The majority of human waste collected and sent through sewage systems that is treated in wastewater treatment plants (WWTPs) and discharged into larger water bodies such as rivers, lakes, streams, groundwaters and coastal waters [164, 166, 167]. This treated discharged wastewater can eventually have both potable and non-potable uses in household, recreational and agricultural settings [4, 167]. Throughout the time before the treated wastewater reaches humans, bacteria harboring ARGs in wastewater interacts with millions of other bacteria and throughout these interactions HGT can occur [166, 167]. Waters in the environment containing fecal waste have been seen as a hotspot for ARG transfer, leading to dissemination and proliferation of the genes among a vast array of bacterial species [166, 167].

Small scale studies focused on traditional WWTPs have shown that they cannot achieve 100% removal of ARGs and ARB [164, 166, 168]. WWTPs have also been the indicated as a hotspot for ARG transfer, especially during the activated sludge treatment process [130, 166-168]. This process is a step in the wastewater treatment that involves biological treatment with other bacteria [165-169]. Activated sludge is an area where large amounts of bacteria are suspended and mixed with oxygen to biologically metabolize many nutrients in the wastewater [165-170]. This step encourages microbial growth and interaction [168-170]. It has been shown that the activated sludge step in the treatment process can make the bacteria that make it through and be discharged in wastewater effluent more likely to be resistant and more likely to be resistant to multiple

antibiotics [165-170]. Throughout the treatment wastewater treatment process solid waste is accumulated and the solids, containing bacteria and ARGs, are removed from the wastewater and the solids often are used for land application in agriculture for growing crops [166, 169, 170]. The rest of the wastewater can be discharged into many types of water bodies which serve many purposes, some of which will involve direct human contact or ingestion [162, 169, 170]. This can include drinking water, bathing, cooking cleaning and other recreational activities, along with agricultural uses [130, 162, 166, 169, 170]. Currently in the united states WWTPs are not designed to remove ARGs and this should be an area of interest for those involved with designing WWTPs and recommending different treatment processes in the future [130, 165, 171].

Hospital wastewater is a source of special interest regarding ARGs entering the environment due to the bacteria shed from the patients in this setting [130, 164, 165, 171, 172]. Hospital effluent wastewater is believed to be a major contributor to the overall contribution to ARGs entering wastewater with studies showing hospital effluent containing higher amounts and ARB than traditional wastewater [130, 164, 171, 172]. One reason for these higher volumes could be due to a higher frequency of excretion in patients, especially in those with enteric infections that cause diarrhea [171]. These infected patients are likely being treated with antibiotics at the hospital which can select for AR, increasing the likelihood of ARB being shed from this setting [130, 164, 165, 171, 172]. Some hospitals have on site treatment systems for their effluent, aiming at reducing this contribution but it is not yet implemented at all sites [130, 164]

Animal waste from agriculture is also a major source of ARGs in the environment [5, 130]. Animal feces can enter the environment through wastewater or runoff, but the

manure can also be used in land applications such as fertilizer for crop growing [5, 130]. Animals in the agricultural setting are often given high amounts of antibiotics for both medicinal and non-medicinal uses [5, 130]. The frequent and heavy use of antibiotics in livestock acts as a selective pressure for AR which can increase the density of ARGs in livestock manure [5]. Animal manure being used in crop fertilization and irrigation poses a human health risk of exposure to potential ARB [5]. The containment and proper treatment and use of animal manure is being pushed because of the perceived risk of its contribution to environmental AR [5, 130, 165]. Feces from wild animals are also thought to contribute to AR in the environment but the overall contribution is thought to be small [130, 163]. Wild animals such as birds have been known to harbor ARB and birds indiscriminately defecate, sometimes onto crop fields or in waters which can lead to new ARGs being transferred or ARB that can eventually be exposed to humans [130, 163].

The *blaTEM* ARG has been researched in wastewater from multiple sources across the globe [173-179]. The *blaTEM* gene began to be researched in wastewater in 2009 to determine the contribution and proliferation of this gene into the receiving water in the United States [176]. The *blaTEM* gene has since been studied in many countries in wastewater samples, including hospital wastewater [173-179]. All quantifications of *blaTEM* across different wastewater samples in different regions found this gene to be present in high levels, especially in hospital samples [173-179]. Studies have quantified the number of *blaTEM* genes in both influent and effluent wastewaters and though there were log reductions from the treatment process in the genes there were still high values of *blaTEM* genes being released from WWTPs [173, 176-179].

The *blaCMY-2* gene began to be researched in swine fecal samples in the environment in Denmark in 2015 [180]. The *blaCMY-2* gene is often associated with agriculture and livestock and is even frequently detected in the NARMS surveillance system [142, 180-183]. *blaCMY-2* has also been detected in wastewater in multiple countries across the globe [184-186]. *blaCMY-2* has been detected in high levels across these study sites, especially in the livestock studies, and the wastewater studies also suggest high levels of *blaCMY-2* in wastewater is likely due to animal sources in the wastewater [181-185]. These studies have also shown that WWTPs do not effectively remove *blaCMY-2* before being discharged into the environment [183-185].

The *OXA-48* gene was first researched in the clinical setting due to its relevance to nosocomial infections [187]. This gene is both hospital and municipal wastewater was determined to be present, widespread and in pathogenic bacteria [184, 188-191]. Studies have shown that the *OXA-48* gene has been able to make it through the WWTP treatment process, being discharged into the environment at levels as high as 1.59×10^{6} copies/ml of hospital effluent [184, 188, 191].

The *KPC* gene was first researched in the clinical setting due to its relevance to nosocomial infections [187, 192-194]. The *KPC* gene has been found in pathogenic bacteria in municipal and hospital wastewater along with surface waters [187, 189, 191-195]. Studies have shown that the *KPC* gene has been able to make it through the WWTP treatment process, being discharged into the environment at levels as high as 1.27×10^{8} copies/ml of hospital effluent. However, it has been shown the wastewater treatment process to be extremely effective at removing the *KPC* gene [192].

The *NDM* gene was first researched in the clinical setting due to its relevance to the associated serious health outcomes from infections of bacteria harboring the gene [187, 189, 191, 195-198]. The *NDM* gene has been found in pathogenic bacteria in municipal and hospital wastewater along with surface waters, though hospital effluent has been a site of special interest for this gene [187, 189, 191, 194-198]. Studies have shown that the *NDM* gene has been able to make it through the WWTP treatment process, being discharged into the environment at levels as high as 1,000 copies/ml of hospital effluent [189, 191, 194, 198, 199]. This gene has even been found in pathogenic bacteria in drinking water samples [196, 197]. *NDM* was also found to be present in environmental samples taken from discharged waste that was produced by pharmaceutical manufacturers in India [196].

The *mcr-1* gene was first researched in the environment due to its low clinical prevalence and serious health outcomes that are related to colistin resistant bacterial infections [63, 200]. The *mcr-1* gene has been found in pathogenic bacteria in municipal and hospital wastewater, along with surface and well waters [201-205]. Studies have shown that the *mcr-1* gene has been able to make it through the WWTP treatment process, being discharged into the environment at levels as high as 10^{10} ARG copies/L of wastewater [200-202, 204-206]. One study in Spain showed an overall increase in the concentration of the *mcr-1* gene in influent and treated wastewater, but showed that the treatment process was able to make a reduction of about 2 log of the *mcr-1* gene [202].

The *mecA* gene was first researched in the clinical setting due to its relevance to MRSA infections and the importance of this gene in conferring resistance in gram-positive bacteria [179, 207-212]. The *mecA* gene has also recently implicated swine farms

as a major source of *mecA* dissemination in the environment [208, 211]. Studies have shown that the *mecA* gene has been able to make it through the WWTP treatment process, being discharged into the environment at levels as high as 10^2 cell equivalents/100ml of wastewater [179, 207-210]. The *mecA* gene has not been found to be ubiquitous among wastewater globally [208, 209].

The *sul1* gene was first researched in the activated sludge step of the wastewater treatment process due to its potential use in agriculture, becoming a potential pathway into the environment [168]. The *sul1* gene has further been implicated as a major ARG in agricultural water use and agricultural waste [213, 214]. Wastewater treatment processes have been found to reduce *sul1* genes considerably, but they have not been completely effective of removing the gene from the effluent waters [166, 178, 179, 213, 215]. The *sul1* gene has been found to be present in effluent wastewater at levels as high as 8 log copies/ml of wastewater [213, 215, 216].

The *tetW* gene was first studied in cattle feedlot lagoons due the high use of tetracycline in agriculture and the possible use of the gene as an indicator of agricultural fecal pollution and source of tetracycline resistant bacteria [217]. Additional studies have looked further into agricultural feedlots, determining that they are a specific area of interest in the accumulation of the *tetW* gene [218, 219]. Wastewater treatment processes have been found to reduce *tetW* genes considerably, but they have not been completely effective of removing the gene from the effluent waters [215, 220, 221]. The *tetW* gene has been found to be present in effluent wastewater at levels as high as 10^6 log copies/100 ml of wastewater [214, 219-224].

The *vanA* gene was first researched in wastewater due to its important clinical importance in gram positive infections, along with its increasing prevalence in agricultural meats [164]. This gene has been studied in more detail in the hospital setting because of the great clinical importance of vancomycin resistant bacteria [192, 225, 226]. Wastewater treatment has been shown to be effective at removing the *vanA* gene from wastewater, though it does not completely remove the gene from the effluent [177, 179, 192, 199, 214, 225, 226]. The *vanA* gene has been found in wastewater effluents at levels as high as 10,000 gene copies/ng of DNA [177].

Abiotic drivers for selection and expression of ARGs include antibiotics, heavy metals and biocides [119]. Antibiotics and antibiotic residues released into the environment are the major selective pressure for AR [163, 165, 167, 171]. The source of antibiotics getting into the environment is the clinical, community, agricultural and manufacturing setting [163, 167]. The major route for antibiotics entering the environment is through wastewater [163, 165, 167, 171]. Antibiotic discharges into wastewater can be from unused antibiotics being flushed from humans, excreted unmetabolized from humans or discharged from manufacturing plants [130, 163, 165, 171]. Often when antibiotics are released into the environment and wastewater it becomes diluted into sublethal concentrations [130, 163, 165, 171]. Exposure to sublethal concentrations of antibiotics is a tremendous selection pressure of AR in bacteria [130, 163, 165, 171]. There have been many studies aimed at determining if there is a correlation between antibiotics and ARGs or ARB in wastewater [172, 177, 227-229]. Multiple studies looking at the relationship between antibiotics and antibiotic residues and ARGs and ARB in hospital wastewater was able to detect a positive correlation

between the two [172, 177, 227, 228]. These correlations have supported the hypothesis that antibiotics being discharged into the environment are selecting for ARB [130, 167]. The solution being proposed to reduce this selection is through proper treatment and disposal of antibiotics before entering the environment [130, 162, 163, 165, 167].

Heavy metals have antibacterial properties targeting multiple sites of the bacterial cells [230-234]. Metals that are most commonly used include mercury, silver, copper, gold, tellurium, potassium, magnesium and zinc salts [230-234]. Metals are used clinical settings during surgeries, used in medicines, and they are also used in agriculture and animal husbandry as growth promoters, fungicides, herbicides and antibiotics [230-234]. Heavy metals are sometimes preferred in agriculture in food as an alternative to traditional antibiotics [230-234]. These metals are also released into the environment through many settings such as occupational, agricultural manufacturing and mining operations [230-234]. Bacteria have developed genes to provide resistance to these metals just as they have developed resistance to antibiotics [230-234]. These genes that code for heavy metal resistance are found on mobile genetic elements such as plasmids, integrons and transposons, just as ARGs [230-234]. It has been suggested that bacteria containing heavy metal resistant genes often carry ARGs on the same mobile genetic elements and there is co-selection for metal resistance and AR in bacteria [230-234]. These heavy metals are insoluble and are extremely stable in soils, allowing them to persist in the environment for long periods of time [230-234]. This stability and accumulation in the environment have allowed for prolonged exposure and persistent selection of resistance for both the metals and AR [230-234]. Studies have shown positive correlations between

metals in the environment and the quantity of ARGs, further supporting the idea of heavy metals as a source of AR selection [230-234].

Biocides are chemical compounds "intended to destroy, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means" [235]. Biocides are most commonly associated with household surface cleaners such as antimicrobial wipes and sprays that can be bought at most general stores [235]. Biocides are also used in healthcare facilities on hospital equipment, used directly on humans, food products, food preservatives, animal husbandry and in water treatment systems [235, 236]. The most important environmental biocide is chlorine which is used globally in wastewater and drinking water treatment systems [235, 236]. Bacteria resistant to chlorine are thought to confer resistance through the use of efflux pumps [235, 236]. This mechanism for chlorine resistance in bacteria helps co-select for antibiotic resistance [235-237]. One study has shown an increase in ARB after chlorination in a wastewater treatment system [236]. A drinking water study also displayed an increase in AR *Pseudomonas aeruginosa* after chlorination of drinking water [237]. The wide use of chlorine in wastewater and drinking water systems create a risk for selection of ARGs and ARB in our drinking and wastewater that has not yet been adequately characterized [235, 237].

2. Methods

2.1 Purpose of Project

The purpose of this study is to provide data on the concentrations of antibiotic resistance genes in influent wastewater samples from urban and rural sites across the

United States. The project aim is to test a panel of 10 clinically significant antibiotic resistance genes (Table 1) to determine the presence and concentrations of these genes in U.S. influent wastewater samples. This project is a descriptive, hypothesis driven study that will provide national data that will help improve water quality, waste management and public health applications.

2.2 Sample Sites and Collection Methods

Influent wastewater samples were collected from 49 different WWTPs throughout the United States. Samples were collected from January 29, 2018 – April 10, 2018. Samples sites were chosen to represent influent wastewater from urban and rural areas. To determine urban and rural designations for each site the estimated surrounding area population based on U.S. Census data was used. A WWTP was considered to be rural if it was in an area with a population >3,500 people and it was at least 50km away from an urban center. WWTPs were considered urban if they were in an area with at least 1,000,000 people. The number of samples from each state and its geographical designation can be seen in Table 2. Plant utility workers collected 1L of influent wastewater and shipped it to EPA in Cincinnati. 25ml from the 1L sample was filtered through a 0.45µm mixed cellulose ester filter and archived at -80°C. Filter blanks were prepared in parallel by filtering 10 ml sterile molecular grade water. Samples were collected in strict accordance with standard methods recommended in the *Standard Methods for the Examination of Water and Wastewater* [238]. Samples were considered eligible for testing if received within 48-hrs of collection and if a shipping temperature of 4°C was maintained during transport. Shipping temperatures were monitored using the

iButton temperature loggers DS1920 (Maxim, Integrated, San Jose, CA), following manufacturer's procedures. Archived frozen filters were sent to the environmental microbiology lab at the Centers for Disease Control and Prevention (CDC) in Atlanta, GA for processing and testing.

2.3 DNA Extraction

DNA was extracted from the filters using the Qiagen Dneasy PowerWater Kit (Qiagen Inc., Germantown, MD), following the manufacturer instructions. One extraction blank using molecular grade water was processed in parallel with each set of filters during the extraction process as a control for cross contamination. Filter blanks (4 total) were processed in parallel with the samples. Positive controls were provided through the CDC and FDA Antibiotic Resistance Isolate Bank [150]. AR isolate bank strains used in the assays are listed in Table 3. No naturally occurring *tetW* positive controls were available. The Gblock standard also served as the positive control for the *tetW* assay. Control strains were incubated overnight in enriched broth, then pelleted and washed before DNA was extracted using the Qiagen Dneasy PowerWater Kit (Qiagen Inc., Germantown, MD).

2.4 DNA Quantification and Dilution

DNA concentrations were determined by using a NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc, Waltham, MA). DNA concentrations from the filter samples ranged from 12.4-199.2 ng/µl. Final DNA

concentrations were adjusted to 5ng/µl in nuclease free water and aliquoted prior to storage. Final DNA samples were stored at -4°C for further analysis.

2.5 PCR Reagents

Primers and probes used in this study are listed in Table 1. All probes incorporated the ZEN double-quenched system (Integrated DNA Technologies, Skokie, IL) and FAM fluorescent dye. TaqMan Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA) was used for all assays except *blaTEM* which detected reagent contamination from the manufacturer. The assay for *blaTEM* used the TaqPath qPCR Master Mix (Applied Biosystems, Foster City, CA).

2.6 Gblock and Standards

A single Gblock construct ARG CDC001 containing 13 ARG targets was used to generate standard curves for quantification. Developing the standard curve for quantification from the Gblock was conducted on each of the five targets in this study. The standard curve was created using 10-fold dilutions starting at 50,000 copies to 5 copies per reaction. All assays yielded an R^2 for targets greater than 0.98. Tests confirmed that the lower limit of detection defined as the lowest standard concentration that yields a positive result. The lower limit of detection for all targets was 5 DNA copies per reaction. The limit for quantification, defined as the lowest standard concentration that yields a reliable Ct value 100% of the time, was 50 DNA copies per reaction.

2.7 Inhibition Check

Inhibition was assessed using the Applied Biosystems TaqMan Exogenous Internal Positive Control Kit (Applied Biosystems, Foster City, CA). A no template control was assessed along with all of the samples. A sample assay with a Ct value difference of 2 or more from the no template control Ct value was considered to be inhibited. Samples were also considered inhibited if no Ct value was produced in the assay.

2.8 Quantitative PCR

Assays included 10ng of extracted DNA samples. For the filter and extraction blanks, 2 µl of undiluted DNA was tested. All samples, filter blanks, and extraction blanks were tested in triplicate against each individual target using MicroAmp Optical 96-Well Reaction Plates (Applied Biosystems, Foster City, CA). Each plate included the 5-point standard curve, positive control, and no template control in triplicate. Cycling conditions of the reactions consisted of denaturing at 95°C for 10 minutes, followed by 45 cycles of: denaturation at 95°C for 15 seconds, annealing, extension, fluorescence capture at 60°C for 1 minute.

2.9 Statistical Analysis

The mean standard curve was used to estimate ARG copies per reaction. Each sample must have had no more than a 2 Ct difference among the triplicate wells to be included in the statistical analysis. A total of 22 observations were removed from the data set because they did not meet this criterion. One of the sample sites was removed completely from the analysis because it did not meet the criteria to be considered an

urban or rural site. This left a total of 468 out a possible 490 values for final analysis. Initial mean copies for each target in each sample were back calculated and transformed to Log10 ARG copies/ml influent wastewater. Samples were categorized by the number of positive wells for each test. If a sample had all three wells test positive it was considered to be positive and quantifiable, samples with one or two positive wells were considered positive but not quantifiable (PNQ) and given a mean value of 2.5 copies, which is half the limit of detection. Samples with no positive wells were considered negative and given a value of 0.5 copies for statistical analysis. Negative samples were given a value of 0.5 because it was known that all samples had fewer than limit of detection in each well, but it is not certain that there are no genes present. Each sample site was grouped into regions using the PulseNet region designations [149]. Statistical tests were conducted using SAS 9.4 (SAS Institute Inc., Cary, NC, USA) using PROC FREQ, MEANS, TTEST and GENMOD. The variance for each t-test was determined to be equal or unequal based on the Folded F test p-value. The Satterthwaite test statistic and p-value were used to analyze the difference in means if the variances were determined unequal. The Pooled test statistic and p-value were used to analyze the difference in means if the variances were determined to be equal. A p-value < 0.05 was used to determine a statistically significant difference in the means. Figures were developed using JMP 14 (SAS Institute Inc., Cary, NC, USA).

3.Results

3.1 Overall Antibiotic Resistance Gene Concentrations

The frequencies of positive, PNQ and negative samples can be seen in Table 4. The *blaCMY-2, blaTEM, tetW* and *sul1* targets were positive in every sample (Table 4). All targets were detected in at least 22% of all samples (Table 4). Negative samples were the most frequent to occur for targets *mcr-1* and *NDM*. All figures used three letter abbreviations to label each gene target and the coding of the abbreviations can be seen in Table 5. *KPC* and *sul1* had the largest ranges in Log10 copies/ml and *sul1* had the highest concentrations of all targets (Figure 1). The targets *mcr-1* and *NDM* had the lowest concentrations of all targets which was expected since these targets are still rarely seen in clinical samples in the United States (Figure 1).

3.2 Urban and Rural Comparisons

There were 229 rural samples and 239 urban samples in the analysis. Table 6 shows the frequency for urban/rural samples by target. The Log10 copies/ml ranges for urban and rural areas by each target can be seen in Figure 2. In rural areas *sul1* had the highest concentrations, and *mcr-1* and *NDM* had the lowest concentrations (Figure 2). In urban areas *tetW* had the highest concentrations while *mcr-1* and *NDM* had the lowest (Figure 2).

T-Tests were conducted to test the mean difference between the urban and rural samples. Table 7 displays the mean difference in urban/rural samples by target. ARG concentrations in urban samples were greater than rural for all targets except for *OXA*, *vanA* and *sul1* (Table 7). Rural samples for the SUL target were greater than urban by 3.2390 Log10 copies/ml (p-value < .0001). The largest difference between urban and rural samples was for target *KPC*, where urban concentrations were greater by 1.3074

Log10 copies/ml (p-value < 0.05). Differences for urban/rural by target were significant for all targets except OXA and VAN.

3.3 Regional Comparisons

Table 8 displays the sample frequency breakdown for each region by each target. The Log10 copies/ml ranges for each region by each target can be seen in Figure 3. The Central region has the largest range and highest concentration of Log10 copies/ml among all regions (Figure 3). There is a large regional variation for *sul1* (Figure 3). *sul1* and *tetW* have the highest regional quantities among the targets and *mcr-1* and *NDM* have the lowest (Figure 3).

The difference in mean of the Log10 copies/ml among regions was calculated for each target. Each region was designated as a reference group to test the difference in the means for each target. Table 9 shows the test results of the regional analysis for target *blaCMY-2*. None of the regional comparisons for target *blaCMY-2* had a p-value < 0.05, failing to be considered statistically significant. Table 10 shows the test results of the regional analysis for target *KPC*. None of the regional comparisons for target *KPC* had a p-value < 0.05, failing to be considered statistically significant. Table 11 shows the test results of the regional analysis for target *mcr-1*. None of the regional comparisons for target *mcr-1* had a p-value < 0.05, failing to be considered statistically significant. Table 12 shows the test results of the regional analysis for target *mecA*. None of the regional comparisons for target *mecA* had a p-value < 0.05, failing to be considered statistically significant. Table 13 show the test results of the regional analysis for target *NDM*. The comparison of means from the Mid Atlantic, Midwest and Northeast all compared to the Mountain region were determined to be statistically significant (p-value < 0.05). The

Mountain region was greater than all three of these regions in these tests. No other comparisons for the *NDM* target were statistically significant. Table 14 shows the test results of the regional analysis for target *OXA-48*. The comparison of mean between the Central and West region was the only test that had statistical significance (p-value < 0.05). The Central region was greater than the West by 1.5612 Log10 copies/ml. No other comparisons for the *OXA-48* target were statistically significant. Table 15 shows the test results of the regional analysis for target *sul1*. Ten of the regional comparison differences for target *sul1* had a p-value < 0.05 and were determined to be significantly significant. The greatest statistically significant difference for the *sul1* target was between the Midwest and Central regions, where the Central region was greater than the Midwest by 3.4010 Log10 copies/ml (Table 15). Table 16 shows the test results of the regional analysis for target *blaTEM*. None of the regional comparisons for target *blaTEM* had a p-value < 0.05, failing to be considered statistically significant. Table 17 shows the test results of the regional analysis for target *tetW*. The only statistically significant differences for target *tetW* were in the comparison against the West region where the Central, Mid Atlantic, Midwest and Mountain region all had statistical differences. The greatest statistically significant difference for the *tetW* target was between the West and Midwest regions, where the West region was greater than the Midwest by 0.5538 Log10 copies/ml (Table 17). Table 18 shows the test results of the regional analysis for target *vanA*. None of the regional comparisons for target *vanA* had a p-value < 0.05, failing to be considered statistically significant.

4. Discussion

4.1 Discussion

The concentrations of ARGs varied significantly across regions of the United States and among urban and rural areas in these regions. There are anthropogenic influences on the presence and quantities of the tested ARGs that possibly have an impact on the health of those people living in these areas. There are also many environmental variables that likely have large influences on the presence of the tested ARGs, along with other genes that were not in this study. The high levels of the β-lactam resistant genes and the fact that β-lactams are the most widely used antibiotics today suggest a relationship between the clinical use and the environmental levels of the genes encoding resistance to the drugs [127]. The extremely large range of the *sul1* gene concentration and that the highest levels of this gene being found in rural areas could point to the importance of agriculture in the dissemination of this gene in the environment [213, 214]. A similar outcome was observed with the *vanA* gene, also supporting the idea that agriculture is playing a major role in the dissemination of this gene [164, 180]. Tetracycline is a common antibiotic often the first choice for treatment options and this could explain the high levels of the *tetW* gene across all samples in this study [127]. Most AR studies conducted before this one was in other countries around the globe, showing that this issue is more common around the globe than expected. It also shows the homogeny in the antibiotics we use and that the sources of the genes that get into the environment are often the same.

Positive *mcr-1* samples were only found in the following states: California, Florida, New Jersey, Ohio, Pennsylvania, Texas, Virginia and Washington. All of the positive *mcr-1* samples were from urban areas. Human clinical isolates were identified by the CDC in all of these states except for Florida and Ohio. The most recent of these

identified human isolate was in July of 2018 from California. The *mcr-1* gene being found in states with no reported cases shows how there can often be a gap between clinical surveillance and the environmental circulation of resistant bacteria. The *mcr-1* gene being found in states without any CDC confirmed human or animal isolates also stresses the urgency of surveillance of the *mcr-1* gene in the clinic and the environment to discover and mitigate the risk before outbreaks of pathogens harboring the gene occurs.

The *sul1* target was only target that had a significantly higher concentrations in rural areas and also had the largest mean difference between urban and rural areas. This large difference in rural areas is expected, as agricultural sources in rural areas have been found to be a major contributor to *sul1* in the environment [213, 214]. All other targets had higher concentrations in the urban areas than rural. This may be because urban areas have a greater populations and diversities of people, mixing more sick people and more people coming from different areas around the world. The carbapenemase gene targets all have lower concentrations in rural areas. Carbapenems are widely used antibiotics and common for people with bacterial infections. Carbapenem resistance is also relatively new and we would expect to see it in high density areas first, then spread to low density areas.

The Mountain and West region showed significant difference in a few of the targets and the reason for this is not completely evident. The Mountain region might have significant differences due to agriculture in the Mountain states. The odds of finding positive samples in the Mountain region also suggest there is a major source or driver in the Mountain states that is leading to more ARGs in their wastewater. The West region

was composed of all coastal states, states that take in a lot of travelers and tourists, which could bring many different bacteria and ARGs along with them.

It is increasingly clear that anthropogenic inputs are impacting environmental AR. Several ARGs have been suggested as indicators for environmental AR. The *tetW* gene, however, would be a poor candidate as an environmental AR indicator based on our results due to the consistently high concentrations across all samples and regions. Ideally, an environmental surveillance marker would need to have a large variation among many different settings and regions in the United States. This variation would be useful in estimating the level of contamination and risk of other genes or bacteria being present in those same samples. *KPC*, *OXA*, *sul1* and *vanA* all showed potential as possible environmental surveillance markers based on the results of this study.

4.2 Limitations

There are several important limitations in this study. The samples are from a single point in time, without replication. The sample sites of this study are not completely representative of the United States. There were many states that samples were not collected from. Since the site selection was based on urban and rural areas there was a large gap in sites that could be described as suburban and have populations that fell in between the selection criteria for the urban and rural sites. The collected samples were not representative of those that use septic tanks in the United States. The study only used qPCR methods to identify and quantify the genes in our samples, so we were not able to determine how many of these genes were in viable or pathogenic bacteria. The methods used for our assays were not able to determine if there were multiple copies of a gene in one cell since genes found on plasmids could carry multiple copies of the ARG.

4.3 Conclusion

Urban areas are a major area of high environmental ARG concentrations. The Mountain and West region displayed significant differences of some ARG targets compared to other regions. The *mcr-1* gene target is one of potential concern and the results suggest that there is a present risk of bacteria acquiring the gene in the environment or of people being exposed to bacteria harboring the gene. Further research is needed on these targets in more localized studies to determine factors related to release, dissemination and risk of exposure in the environment. Active environmental surveillance on ARGs in the United States should become a top priority when thinking about how we can help reduce the risk and propagation of AR in our environment and prevent ARB exposure and infections in humans and animals in the future.

4.4 Recommendations

Future research in this area should include testing to determine AR in viable and pathogenic bacteria. Similar studies should also be conducted as a follow up to this study but more concentrated in specific regions of the United States. These concentrated studies could provide more precise insight on the sources and factors that contribute to these ARGs in the environment. Additional source tracking should be conducted to determine major sources, especially in urban areas. Correlation studies could also be conducted looking at environmental ARG levels and number of clinical AR cases that are related to those ARGs. This would help in providing information on the link between the environment and human health outcomes. WWTP studies should also be considered. WWTP studies focusing on the effectiveness of the different WWTP processes on removing ARGs and the difference between influent and effluent level, while also

determining the proportion of bacteria in the effluent that harbor ARGs. Risk assessment studies looking at environmental ARGs and risk to human exposure and health should be considered.

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6.Figures and Tables

Table 1: Target assay primers and probes

Target	Sequence	Product Length (bp)	Reference
blaTEM		84	[176]
Forward Primer	CACTATTCTCAGAATGACTTGGT		
Reverse Primer	TGCATAATTCTCTTACTGTCATG		
Probe	CCAGTCACAGAAAAGCATCTTACGG		
blaCMY-2		128	[180]
Forward Primer	AGACGTTTAACGGCGTGTTG		
Reverse Primer	TAAGTGCAGCAGGCGGATAC		
Probe	TATCGCCCGCGGCGAAAT		
OXA-48		297	[187]
Forward Primer	GATTATGGTAATGAGGACATTTCGGGC		
Reverse Primer	CATATCCATATTCATCGCAAAAAACCACAC		
Probe	CCATTGGCTTCGGTCAGCATGGCTTGTTT		
KPC		184	[187]
Forward Primer	GCAGCGGCAGCAGTTTGTTGATT		
Reverse Primer	GTAGACGGCCAACACAATAGGTGC		
Probe	CAGTCGGAGACAAAACCGGAACCTGC		
NDM		207	[187]
Forward Primer	CCAGCAAATGGAAACTGGCGAC		
Reverse Primer	ATCCAGTTGAGGATCTGGGCG		
Probe	ACCGAATGTCTGGCAGCACACTTC		
mcr-1		116	[200]
Forward Primer	CATCGCGGACAATCTCGG		
Reverse Primer	AAATCAACACAGGCTTTAGCAC		
Probe	AACAGCGTGGTGATCAGTAGCAT		

Table 1: *Target assay primers and probes*

Target	Sequence	Product Length (bp)	Reference
mecA		135	[212]
Forward Primer	AACCACCCAATTTGTCTGCC		
Reverse Primer	TGATGGTATGCAACAAGTCGTAAA		
Probe	CCTTGTTTCATTTTGAGTTCTGCAGTACCGG		
sul1		67	[168]
Forward Primer	CCGTTGGCCTTCCTGTAAAG		
Reverse Primer	TTGCCGATCGCGTGAAGT		
Probe	CGAGCCTTGCGGCGG		
tetW		66	[217]
Forward Primer	GCAGAGCGTGGTTCAGTCT		
Reverse Primer	GACACCGTCTGCTTGATGATAAT		
Probe	TTCGGGATAAGCTCTCCGCCGA		
vanA		65	[164]
Forward Primer	CTGTGAGGTCGGTTGTGCG		
Reverse Primer	TTTGGTCCACCTCGCCA		
Probe	CAACTAACGCGGCACTGTTTCCCAAT		

Table 2: Sample number by state and urban/rural designation

State	Region	Number of Urban Sites	Number of Rural Sites	Total Number of Sites
Alabama	Southeast	0	1	1
Arizona	Mountain	1	0	1
Arkansas	Central	0	1	1
California	West	2	1	3
Delaware	Mid Atlantic	1	0	1
Florida	Southeast	1	0	1
Georgia	Southeast	1	1	2
Idaho	Mountain	0	2	2
Illinois	Midwest	3	0	3
Indiana	Midwest	0	1	1
Louisiana	Southeast	0	1	1
Maine	Northeast	0	2	2
Massachusetts	Northeast	1	0	1
Michigan	Midwest	2	0	2
Missouri	Central	0	1	1
Montana	Mountain	0	1	1
New Jersey	Northeast	1	0	1
New York	Northeast	0	3	3
North Carolina	Mid Atlantic	0	1	1
North Dakota	Central	0	1	1
Ohio	Midwest	3	0	3
Oklahoma	Central	0	2	2
Oregon	West	1	0	1
Pennsylvania	Mid Atlantic	2	0	2
Tennessee	Southeast	0	1	1
Texas	Mountain	3	0	3
Vermont	Northeast	0	1	1
Virginia	Mid Atlantic	1	0	1
Washington	West	2	0	2
West Virginia	Mid Atlantic	0	2	2
Wisconsin	Midwest	0	1	1

Table 3: Target assay positive controls

PCR assay	Organism	Strain
blaTEM	*Salmonella senftenberg*	AR0405
blaCMY-2	*Salmonella typhimurium*	AR0408
OXA-48	*Klebsiella pneumoniae*	AR0160
KPC	*Klebsiella oxytoca*	AR0147
NDM	*Escherichia coli*	AR0118
mcr-1	*Escherichia coli*	AR0346
mecA	*Staphylococcus aureus*	AR0683
sul1	*Salmonella concord*	AR0407
tetW	N/A	gBlock standard
vanA	*Enterococcus avium*	AR0571

Table 4: Sample frequency by target

Sample Frequency by Target				
Target	**Negative (% of Total)**	**Positive Not Quantifiable (PNQ) (% of Total)**	**Positive and Quantifiable (% of Total)**	**Total**
blaCMY-2	0 (0)	0 (0)	49 (100)	49
KPC	3 (6.25)	4 (8.33)	41 (85.42)	48
mcr-1	39 (81.25)	4 (8.33)	5 (10.42)	48
mecA	1 (2.44)	9 (21.95)	31 (75.61)	41
NDM	38 (77.55)	8 (16.33)	3 (6.12)	49
OXA-48	8 (18.60)	10 (23.26)	25 (58.14)	43
sul1	0 (0)	0 (0)	49 (100)	49
blaTEM	0 (0)	0 (0)	48 (100)	48
tetW	0 (0)	0 (0)	48 (100)	48
vanA	4 (8.89)	9 (20)	32 (71.11)	45

Table 5: Three letter abbreviations to label each gene target that are used in all figures

Abbreviations Used for Gene Targets	
Gene Target	**Abbreviation**
blaCMY-2	CMY
KPC	KPC
mcr-1	MCR
mecA	MEC
NDM	NDM
OXA-48	OXA
sul1	SUL
blaTEM	TEM
tetW	TET
vanA	VAN

Figure 1: Overall distribution of ARG concentrations

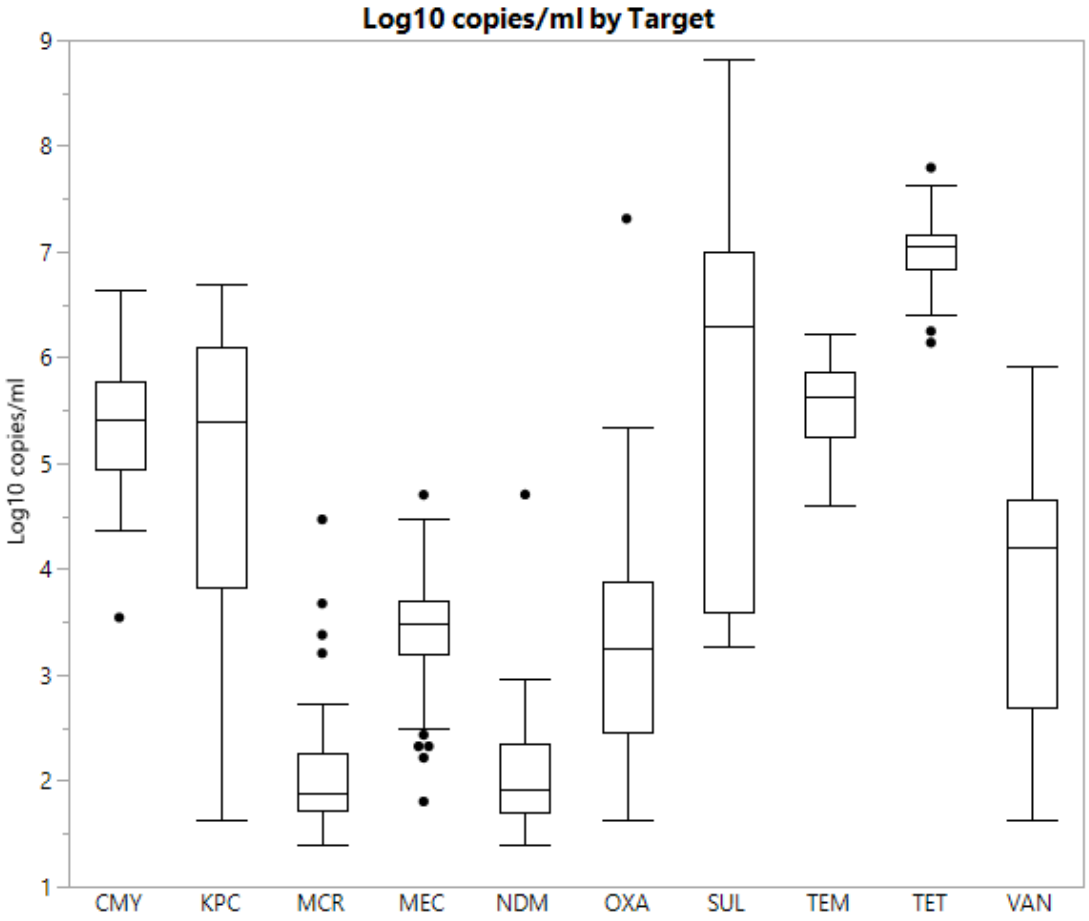

Table 6: Number of urban/rural samples by target

Number of Urban/Rural Samples by Target			
Target	**Rural**	**Urban**	**Total**
blaCMY-2	24	25	49
KPC	23	25	48
mcr-1	24	24	48
mecA	21	20	41
NDM	24	25	49
OXA-48	22	21	43
sul1	24	25	49
blaTEM	24	24	48
tetW	23	25	48
vanA	20	25	45
Total	229	239	468

Figure 2: Distribution of ARG concentrations by urban/rural designation

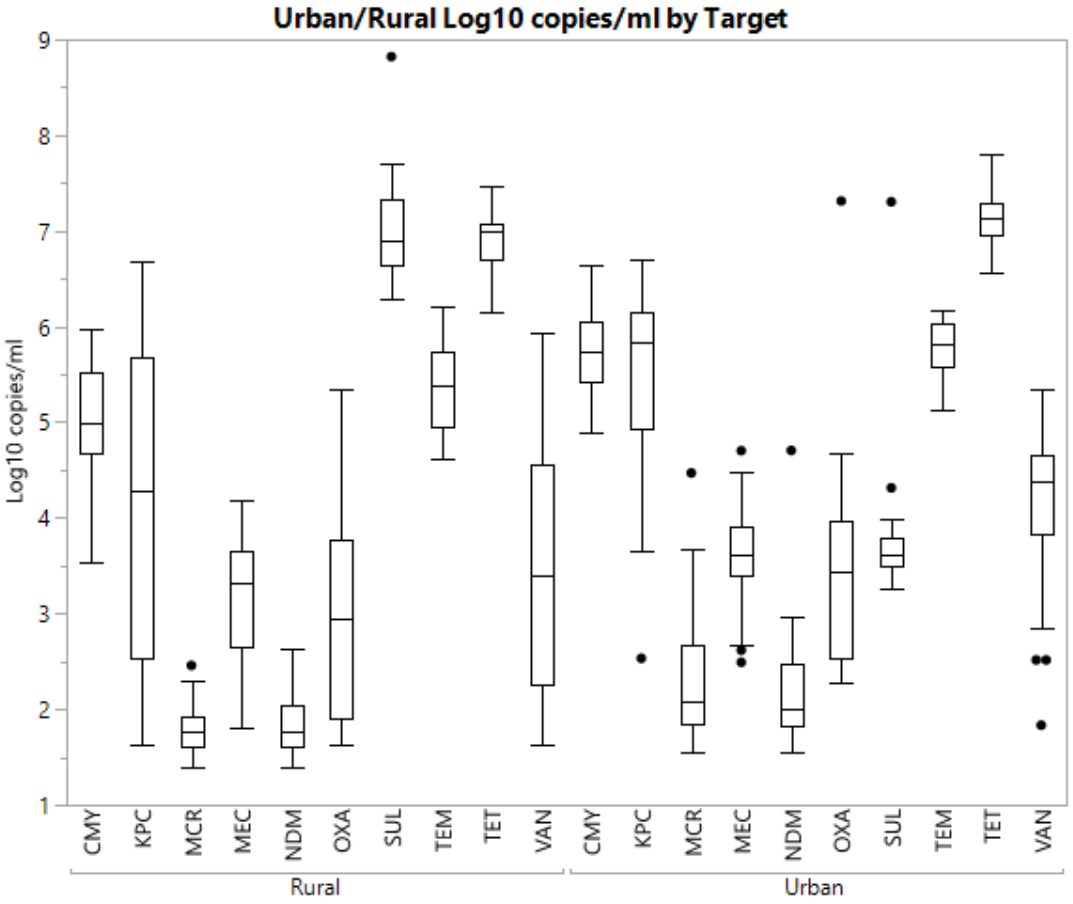

Table 7: T-Test statistics of rural-urban Log10 copies/ml mean differences by target

Urban/Rural Log10 copies/ml Mean Difference by Target					
Target	**Variance**	**Mean Difference (Rural – Urban)**	**95% CL Lower Limit**	**95% CL Upper Limit**	**P-Value**
blaCMY-2	Equal	-0.6964	-0.9911	-0.4018	<.0001
KPC	Unequal	-1.3074	-2.1091	-0.5058	0.0021
mcr-1	Equal	-0.5822	-0.8936	-0.2709	0.0005
mecA	Equal	-0.4159	-0.7905	-0.0413	0.0305
NDM	Unequal	-0.3602	-0.6633	-0.0572	0.0211
OXA-48	Equal	-0.4626	-1.1467	0.2216	0.1795
sul1	Equal	3.2390	2.8528	3.6252	<.0001
blaTEM	Unequal	-0.4225	-0.6408	-0.2042	0.0003
tetW	Equal	-0.2653	-0.4499	-0.0807	0.0058
vanA	Equal	-0.6492	-1.3217	0.0233	0.0581

Table 8: Number of samples by region and target

Number of Samples by Region and Target								
Target	**Region**							
	Central	**Mid Atlantic**	**Midwest**	**Mountain**	**North -east**	**South -east**	**West**	**Total**
blaCMY-2	5	7	10	7	8	6	6	49
KPC	5	7	10	7	8	6	5	48
mcr-1	5	7	10	7	8	6	5	48
mecA	5	6	9	6	6	6	3	41
NDM	5	7	10	7	8	6	6	49
OXA-48	5	7	8	6	7	5	5	43
sul1	5	7	10	7	8	6	6	49
blaTEM	5	7	10	7	7	6	6	48
tetW	5	7	10	7	7	6	6	48
vanA	4	6	9	7	7	6	6	45
Total	49	68	96	68	74	59	54	468

Figure 3: Distribution of AR gene concentrations by PulseNet region

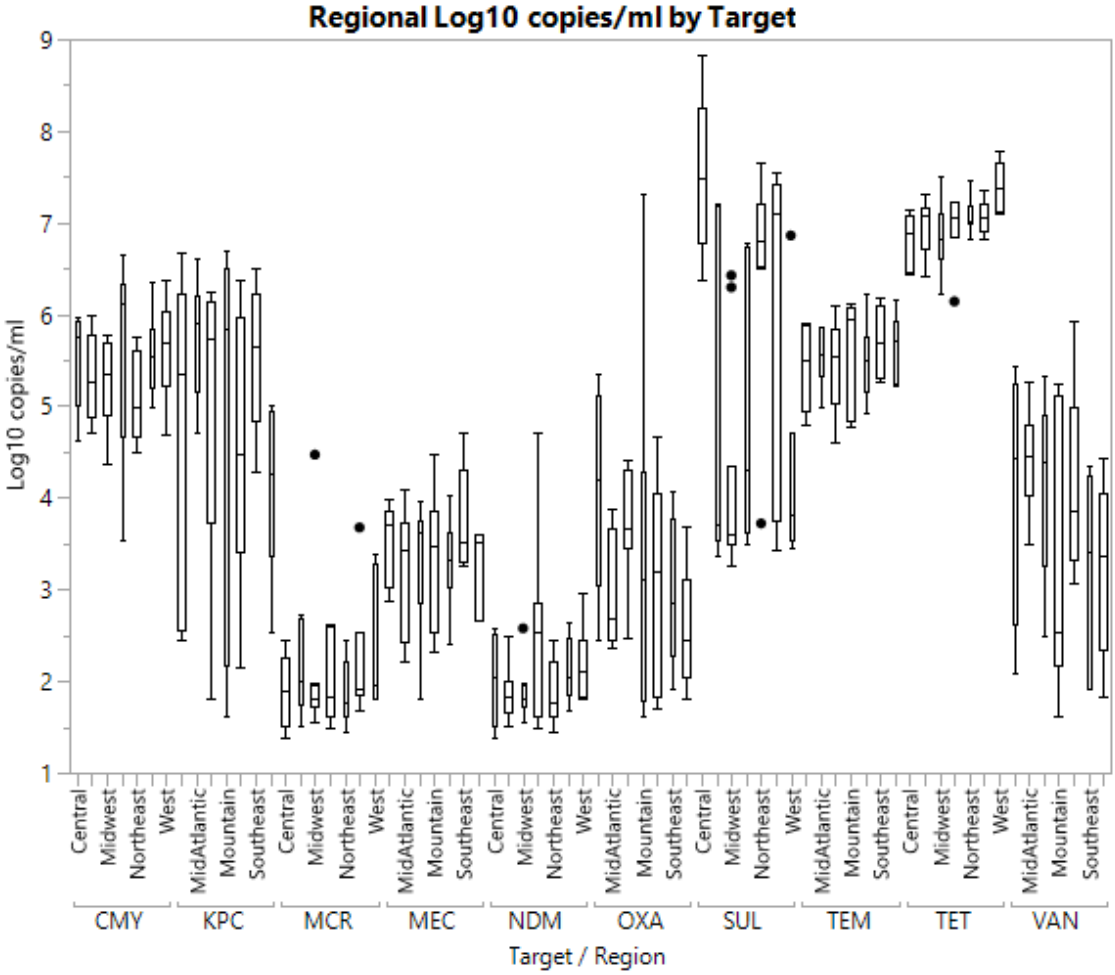

Table 9: Analysis of regional Log10/ml mean differences for target blaCMY-2

Regional Log10/ml Mean Differences for Target *blaCMY-2*				
Region	**Mean Difference Estimate**	**Confidence Limits**		**P-Value**
Mid Atlantic v. Central	-0.1529	-0.8781	0.5724	0.6795
Midwest v. Central	-0.2842	-0.9627	0.3942	0.4116
Mountain v. Central	-0.0575	-0.7827	0.6678	0.8766
Northeast v. Central	-0.4140	-1.1201	0.2922	0.2505
Southeast v. Central	0.0371	-0.7130	0.7871	0.9228
West v. Central	0.1069	-0.6431	0.8569	0.7800
Midwest v. Mid Atlantic	-0.1314	-0.7418	0.4790	0.6732
Mountain v. Mid Atlantic	0.0954	-0.5667	0.7575	0.7777
Northeast v. Mid Atlantic	-0.2611	-0.9022	0.3799	0.4247
Southeast v. Mid Atlantic	0.1899	-0.4992	0.8790	0.5891
West v. Mid Atlantic	0.2598	-0.4293	0.9489	0.4600
Mountain v. Midwest	0.2268	-0.3836	0.8372	0.4666
Northeast v. Midwest	-0.1297	-0.7173	0.4578	0.6652
Southeast v. Midwest	0.3213	-0.3183	0.9609	0.3249
West v. Midwest	0.3911	-0.2485	1.0308	0.2307
Northeast v. Mountain	-0.3565	-0.9975	0.2846	0.2757
Southeast v. Mountain	0.0945	-0.5946	0.7836	0.7880
West v. Mountain	0.1644	-0.5247	0.8535	0.6401
Southeast v. Northeast	0.4510	-0.2179	1.1200	0.1863
West v. Northeast	0.5209	-0.1481	1.1898	0.1270
West v. Southeast	0.0698	-0.6453	0.7850	0.8482

Table 10: Analysis of regional Log10/ml mean differences for target KPC

Regional Log10/ml Mean Differences for Target *KPC*				
Region	**Mean Difference Estimate**	**Confidence Limits**		**P-Value**
Mid Atlantic v. Central	1.1450	-0.5626	2.8526	0.1888
Midwest v. Central	0.3705	-1.2268	1.9679	0.6493
Mountain v. Central	-0.0976	-1.8052	1.6100	0.9108
Northeast v. Central	-0.0911	-1.7536	1.5715	0.9145
Southeast v. Central	0.9500	-0.8159	2.7159	0.2917
West v. Central	-0.4110	-2.2554	1.4335	0.6623
Midwest v. Mid Atlantic	-0.7745	-2.2116	0.6627	0.2909
Mountain v. Mid Atlantic	-1.2426	-2.8014	0.3162	0.1182
Northeast v. Mid Atlantic	-1.2361	-2.7454	0.2732	0.1085
Southeast v. Mid Atlantic	-0.1950	-1.8175	1.4275	0.8138
West v. Mid Atlantic	-1.5560	-3.2636	0.1517	0.0741
Mountain v. Midwest	-0.4682	-1.9053	0.9690	0.5232
Northeast v. Midwest	-0.4616	-1.8450	0.9217	0.5131
Southeast v. Midwest	0.5795	-0.9265	2.0854	0.4508
West v. Midwest	-0.7815	-2.3788	0.8158	0.3376
Northeast v. Mountain	0.0065	-1.5028	1.5159	0.9932
Southeast v. Mountain	1.0476	-0.5748	2.6701	0.2057
West v. Mountain	-0.3133	-2.0209	1.3943	0.7191
Southeast v. Northeast	1.0411	-0.5339	2.6161	0.1951
West v. Northeast	-0.3199	-1.9824	1.3427	0.7061
West v. Southeast	-1.3610	-3.1269	0.4049	0.1309

Table 11: Analysis of regional Log10/ml mean differences for target mcr-1

Regional Log10/ml Mean Differences for Target *mcr-1*				
Region	**Mean Difference Estimate**	**Confidence Limits**		**P-Value**
Mid Atlantic v. Central	0.2649	-0.4504	0.9802	0.4680
Midwest v. Central	0.1827	-0.4864	0.8518	0.5926
Mountain v. Central	0.1354	-0.5799	0.8507	0.7106
Northeast v. Central	-0.0080	-0.7044	0.6885	0.9821
Southeast v. Central	0.3310	-0.4088	1.0707	0.3805
West v. Central	0.5569	-0.2158	1.3295	0.1578
Midwest v. Mid Atlantic	-0.0822	-0.6842	0.5198	0.7890
Mountain v. Mid Atlantic	-0.1295	-0.7825	0.5235	0.6976
Northeast v. Mid Atlantic	-0.2729	-0.9052	0.3594	0.3976
Southeast v. Mid Atlantic	0.0661	-0.6136	0.7458	0.8489
West v. Mid Atlantic	0.2920	-0.4234	1.0073	0.4237
Mountain v. Midwest	-0.0473	-0.6493	0.5548	0.8777
Northeast v. Midwest	-0.1907	-0.7702	0.3888	0.5190
Southeast v. Midwest	0.1483	-0.4826	0.7792	0.6450
West v. Midwest	0.3742	-0.2950	1.0433	0.2731
Northeast v. Mountain	-0.1434	-0.7757	0.4889	0.6566
Southeast v. Mountain	0.1956	-0.4841	0.8752	0.5728
West v. Mountain	0.4214	-0.2939	1.1368	0.2482
Southeast v. Northeast	0.3390	-0.3208	0.9987	0.3139
West v. Northeast	0.5648	-0.1316	1.2613	0.1119
West v. Southeast	0.2259	-0.5139	0.9656	0.5495

Table 12: Analysis of regional Log10/ml mean differences for target mecA

Regional Log10/ml Mean Differences for Target *mecA*				
Region	**Mean Difference Estimate**	**Confidence Limits**		**P-Value**
Mid Atlantic v. Central	-0.2877	-1.0570	0.4815	0.4635
Midwest v. Central	-0.1759	-0.8845	0.5328	0.6267
Mountain v. Central	-0.1630	-0.9323	0.6063	0.6780
Northeast v. Central	-0.1997	-0.9690	0.5696	0.6109
Southeast v. Central	0.2529	-0.5164	1.0222	0.5194
West v. Central	-0.2343	-1.1621	0.6935	0.6206
Midwest v. Mid Atlantic	0.1119	-0.5577	0.7815	0.7433
Mountain v. Mid Atlantic	0.1248	-0.6087	0.8582	0.7389
Northeast v. Mid Atlantic	0.0880	-0.6454	0.8215	0.8140
Southeast v. Mid Atlantic	0.5406	-0.1929	1.2741	0.1486
West v. Mid Atlantic	0.0534	-0.8449	0.9517	0.9072
Mountain v. Midwest	0.0129	-0.6567	0.6825	0.9699
Northeast v. Midwest	-0.0238	-0.6934	0.6457	0.9444
Southeast v. Midwest	0.4287	-0.2408	1.0983	0.2095
West v. Midwest	-0.0585	-0.9054	0.7885	0.8924
Northeast v. Mountain	-0.0367	-0.7702	0.6968	0.9218
Southeast v. Mountain	0.4159	-0.3176	1.1494	0.2665
West v. Mountain	-0.0713	-0.9697	0.8270	0.8763
Southeast v. Northeast	0.4526	-0.2809	1.1861	0.2265
West v. Northeast	-0.0346	-0.9330	0.8637	0.9398
West v. Southeast	-0.4872	-1.3856	0.4111	0.2878

Table 13: Analysis of regional Log10/ml mean differences for target NDM

Regional Log10/ml Mean Differences for Target *NDM*				
Region	**Mean Difference Estimate**	**Confidence Limits**		**P-Value**
Mid Atlantic v. Central	-0.1279	-0.7459	0.4900	0.6849
Midwest v. Central	-0.1448	-0.7229	0.4332	0.6233
Mountain v. Central	0.5269	-0.0910	1.1449	0.0947
Northeast v. Central	-0.1478	-0.7495	0.4539	0.6302
Southeast v. Central	0.0967	-0.5424	0.7357	0.7669
West v. Central	0.1599	-0.4791	0.7990	0.6238
Midwest v. Mid Atlantic	-0.0169	-0.5370	0.5032	0.9492
Mountain v. Mid Atlantic	0.6549	0.0907	1.2190	0.0229
Northeast v. Mid Atlantic	-0.0199	-0.5661	0.5264	0.9432
Southeast v. Mid Atlantic	0.2246	-0.3626	0.8118	0.4534
West v. Mid Atlantic	0.2879	-0.2993	0.8750	0.3366
Mountain v. Midwest	0.6718	0.1517	1.1919	0.0114
Northeast v. Midwest	-0.0029	-0.5036	0.4977	0.9908
Southeast v. Midwest	0.2415	-0.3035	0.7865	0.3851
West v. Midwest	0.3048	-0.2402	0.8498	0.2730
Northeast v. Mountain	-0.6747	-1.2209	-0.1285	0.0155
Southeast v. Mountain	-0.4303	-1.0175	0.1569	0.1509
West v. Mountain	-0.3670	-0.9542	0.2202	0.2206
Southeast v. Northeast	0.2444	-0.3255	0.8144	0.4006
West v. Northeast	0.3077	-0.2622	0.8777	0.2900
West v. Southeast	0.0633	-0.5460	0.6726	0.8387

Table 14: Analysis of regional Log10/ml Mean Differences for Target OXA-48

Regional Log10/ml Mean Differences for Target *OXA-48*				
Region	**Mean Difference Estimate**	**Confidence Limits**		**P-Value**
Mid Atlantic v. Central	-1.2132	-2.4721	0.0457	0.0589
Midwest v. Central	-0.4055	-1.6312	0.8203	0.5168
Mountain v. Central	-0.7316	-2.0335	0.5703	0.2707
Northeast v. Central	-1.1019	-2.3608	0.1570	0.0862
Southeast v. Central	-1.1225	-2.4823	0.2373	0.1057
West v. Central	-1.5612	-2.9210	-0.2014	0.0244
Midwest v. Mid Atlantic	0.8077	-0.3050	1.9205	0.1548
Mountain v. Mid Atlantic	0.4816	-0.7146	1.6778	0.4300
Northeast v. Mid Atlantic	0.1113	-1.0380	1.2605	0.8495
Southeast v. Mid Atlantic	0.0907	-1.1682	1.3497	0.8877
West v. Mid Atlantic	-0.3480	-1.6069	0.9110	0.5880
Mountain v. Midwest	-0.3261	-1.4873	0.8350	0.5820
Northeast v. Midwest	-0.6965	-1.8092	0.4163	0.2199
Southeast v. Midwest	-0.7170	-1.9427	0.5087	0.2516
West v. Midwest	-1.1557	-2.3814	0.0700	0.0646
Northeast v. Mountain	-0.3703	-1.5665	0.8258	0.5440
Southeast v. Mountain	-0.3909	-1.6928	0.9110	0.5562
West v. Mountain	-0.8296	-2.1315	0.4723	0.2117
Southeast v. Northeast	-0.0205	-1.2795	1.2384	0.9745
West v. Northeast	-0.4592	-1.7182	0.7997	0.4746
West v. Southeast	-0.4387	-1.7985	0.9211	0.5272

Table 15: Analysis of regional Log10/ml mean differences for target sul1

Regional Log10/ml Mean Differences for Target *sul1*				
Region	**Mean Difference Estimate**	**Confidence Limits**		**P-Value**
Mid Atlantic v. Central	-2.4793	-4.1412	-0.8175	0.0035
Midwest v. Central	-3.4010	-4.9555	-1.8465	<.0001
Mountain v. Central	-2.4514	-4.1133	-0.7896	0.0038
Northeast v. Central	-0.9771	-2.5951	0.6408	0.2365
Southeast v. Central	-1.4404	-3.1589	0.2782	0.1004
West v. Central	-3.2629	-4.9815	-1.5444	0.0002
Midwest v. Mid Atlantic	-0.9217	-2.3203	0.4769	0.1965
Mountain v. Mid Atlantic	0.0279	-1.4891	1.5449	0.9712
Northeast v. Mid Atlantic	1.5022	0.0333	2.9711	0.0450
Southeast v. Mid Atlantic	1.0390	-0.5400	2.6179	0.1972
West v. Mid Atlantic	-0.7836	-2.3626	0.7954	0.3307
Midwest v. Mountain	-0.9496	-2.3482	0.4490	0.1833
Northeast v. Mountain	1.4743	0.0054	2.9432	0.0492
Southeast v. Mountain	1.0111	-0.5679	2.5900	0.2095
West v. Mountain	-0.8115	-2.3905	0.7675	0.3138
Northeast v. Midwest	2.4239	1.0777	3.7701	0.0004
Southeast v. Midwest	1.9607	0.4951	3.4262	0.0087
West v. Midwest	0.1381	-1.3275	1.6037	0.8535
Southeast v. Northeast	-0.4632	-1.9960	1.0695	0.5536
West v. Northeast	-2.2858	-3.8186	-0.7530	0.0035
West v. Southeast	-1.8226	-3.4611	-0.1840	0.0293

Table 16: Analysis of regional Log10/ml mean differences for target blaTEM

Regional Log10/ml Mean Differences for Target *blaTEM*				
Region	**Mean Difference Estimate**	**Confidence Limits**		**P-Value**
Mid Atlantic v. Central	0.1420	-0.3701	0.6541	0.5868
Midwest v. Central	0.0204	-0.4586	0.4994	0.9335
Mountain v. Central	0.1967	-0.3153	0.7088	0.4514
Northeast v. Central	0.1028	-0.4093	0.6149	0.6940
Southeast v. Central	0.2729	-0.2567	0.8024	0.3125
West v. Central	0.2207	-0.3088	0.7503	0.4140
Midwest v. Mid Atlantic	-0.1216	-0.5526	0.3094	0.5803
Mountain v. Mid Atlantic	0.0548	-0.4127	0.5222	0.8184
Northeast v. Mid Atlantic	-0.0392	-0.5067	0.4283	0.8694
Southeast v. Mid Atlantic	0.1309	-0.3557	0.6174	0.5980
West v. Mid Atlantic	0.0787	-0.4078	0.5653	0.7511
Mountain v. Midwest	0.1763	-0.2546	0.6073	0.4226
Northeast v. Midwest	0.0824	-0.3486	0.5134	0.7079
Southeast v. Midwest	0.2525	-0.1991	0.7041	0.2732
West v. Midwest	0.2003	-0.2513	0.6519	0.3846
Northeast v. Mountain	-0.0940	-0.5614	0.3735	0.6936
Southeast v. Mountain	0.0761	-0.4104	0.5627	0.7591
West v. Mountain	0.0240	-0.4626	0.5105	0.9230
Southeast v. Northeast	0.1701	-0.3165	0.6566	0.4932
West v. Northeast	0.1179	-0.3686	0.6045	0.6347
West v. Southeast	-0.0522	-0.5571	0.4528	0.8395

Table 17: Analysis of regional Log10/ml mean differences for target tetW

Regional Log10/ml Mean Differences for Target *tetW*				
Region	**Mean Difference Estimate**	**Confidence Limits**		**P-Value**
Mid Atlantic v. Central	0.2003	-0.1565	0.5571	0.2712
Midwest v. Central	0.0610	-0.2727	0.3948	0.7200
Mountain v. Central	0.1622	-0.1946	0.5191	0.3728
Northeast v. Central	0.2897	-0.0671	0.6465	0.1115
Southeast v. Central	0.2809	-0.0881	0.6499	0.1356
West v. Central	0.6148	0.2458	0.9838	0.0011
Midwest v. Mid Atlantic	-0.1393	-0.4396	0.1610	0.3634
Mountain v. Mid Atlantic	-0.0381	-0.3638	0.2877	0.8189
Northeast v. Mid Atlantic	0.0894	-0.2363	0.4151	0.5906
Southeast v. Mid Atlantic	0.0806	-0.2584	0.4197	0.6411
West v. Mid Atlantic	0.4145	0.0755	0.7535	0.0166
Mountain v. Midwest	0.1012	-0.1991	0.4015	0.5089
Northeast v. Midwest	0.2287	-0.0716	0.5290	0.1356
Southeast v. Midwest	0.2199	-0.0948	0.5346	0.1708
West v. Midwest	0.5538	0.2391	0.8684	0.0006
Northeast v. Mountain	0.1275	-0.1983	0.4532	0.4431
Southeast v. Mountain	0.1187	-0.2203	0.4577	0.4926
West v. Mountain	0.4526	0.1136	0.7916	0.0089
Southeast v. Northeast	-0.0088	-0.3478	0.3303	0.9596
West v. Northeast	0.3251	-0.0139	0.6641	0.0602
West v. Southeast	0.3339	-0.0179	0.6857	0.0629

Table 18: Analysis of regional Log10/ml mean differences for target vanA

Regional Log10/ml Mean Differences for Target *vanA*				
Region	**Mean Difference Estimate**	**Confidence Limits**		**P-Value**
Mid Atlantic v. Central	0.3151	-1.1050	1.7352	0.6636
Midwest v. Central	0.0555	-1.2666	1.3775	0.9345
Mountain v. Central	-0.6971	-2.0760	0.6818	0.3218
Northeast v. Central	0.0813	-1.2976	1.4603	0.9080
Southeast v. Central	-0.9014	-2.3215	0.5187	0.2135
West v. Central	-0.8614	-2.2815	0.5587	0.2345
Midwest v. Mid Atlantic	-0.2597	-1.4192	0.8998	0.6607
Mountain v. Mid Atlantic	-1.0122	-2.2362	0.2118	0.1050
Northeast v. Mid Atlantic	-0.2338	-1.4577	0.9902	0.7081
Southeast v. Mid Atlantic	-1.2165	-2.4867	0.0537	0.0605
West v. Mid Atlantic	-1.1765	-2.4467	0.0937	0.0695
Mountain v. Midwest	-0.7526	-1.8613	0.3561	0.1834
Northeast v. Midwest	0.0259	-1.0828	1.1346	0.9635
Southeast v. Midwest	-0.9568	-2.1163	0.2027	0.1058
West v. Midwest	-0.9168	-2.0763	0.2427	0.1212
Northeast v. Mountain	0.7784	-0.3975	1.9544	0.1945
Southeast v. Mountain	-0.2043	-1.4282	1.0197	0.7436
West v. Mountain	-0.1643	-1.3882	1.0597	0.7925
Southeast v. Northeast	-0.9827	-2.2067	0.2413	0.1156
West v. Northeast	-0.9427	-2.1667	0.2813	0.1311
West v. Southeast	0.0400	-1.2302	1.3102	0.9508