Antibiotic Resistance of Aquatic Gram-Negative Bacteria within Metropolitan Areas: Indianapolis and the Eagle Creek Reservoir

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Abstract

Antibiotic resistance has become a growing public health concern, particularly in major metropolitan areas such as Indianapolis. Urban runoff and waste from hospitals and agricultural areas can introduce resistant bacteria into natural waterways, creating opportunities for the environmental dissemination of antibiotic resistance. Many of Indianapolis' waterways and reservoirs are connected through the White River watershed. Eagle Creek, a manmade reservoir within Eagle Creek Park, is part of this watershed and receives runoff from surrounding areas.

This study assessed the antibiotic resistance profiles of Gram-negative bacteria isolated from Eagle Creek. Water samples were collected, vacuum-filtered to remove debris and larger microorganisms, and cultured on rich media. Individual colonies were purified by restreaking, and antibiotic susceptibility was determined using the Kirby-Bauer disk diffusion method with comparison to an Escherichia coli reference standard.

All 72 Gram-negative isolates exhibited resistance to at least one antibiotic, and 95% demonstrated multidrug resistance. The highest susceptibility was observed with gentamicin, to which only 5.6% of isolates were sensitive. The greatest resistance occurred against ampicillin and amoxicillin/clavulanate, with 95.8% and 79.2% of isolates resistant, respectively. Notably, 51.4% of isolates were also resistant to imipenem, a carbapenem often reserved for severe infections.

These findings suggest that urban aquatic environments may serve as environmental reservoirs of antibiotic-resistant bacteria and highlight the importance of continued surveillance of antimicrobial resistance in local water systems. Future studies incorporating bacterial species identification, whole-genome sequencing, and plasmid analysis are needed to better characterize resistance profiles and clarify the potential implications for environmental dissemination and public health.

Keywords: Antibiotic resistance, antimicrobials, gram-negative, bacteria, multidrug resistance

Background and Introduction

Antibiotic resistance among bacteria is a growing and evolving issue affecting healthcare globally, particularly due to multidrug-resistant bacteria. According to the Center for Disease Control (CDC), multidrug-resistance (MDR) refers to microorganisms - typically bacteria - that are resistant to at least one antibiotic or antimicrobial agent in three or more distinct drug classes.[1] MDR infections directly cause over 1.27 million deaths and are associated with nearly 5 million deaths globally each year.[2] Wastewater and runoff from urban hospitals and agricultural sources introduce antibiotic-resistant bacteria into natural waterways.[3,4] This allows nosocomial and artificially resistant bacteria from areas with increased antibiotic administration to intermingle and share antibiotic resistance genes through horizontal gene transfer.[5,6]

Gram-negative bacteria can transfer genetic material horizontally through three mechanisms: transformation of naked genetic material, conjugation between bacteria, and transduction mediated by bacteriophages.[7] These mechanisms permit the transfer of genetic material that confers antibiotic resistance to environmental pathogens, thereby decreasing the effectiveness of antimicrobials used in clinical settings.[8]

Antibiotics kill or inhibit bacteria through various mechanisms, such as the destruction of the bacterial cell wall or membrane, inhibition of metabolic pathways, or prevention of gene and protein synthesis. Antibiotic resistance genes encode mechanisms that can inactivate antibiotics, prevent antibiotic entry into the bacterial cell, or decrease the intracellular concentration of antibiotics.[9] Specifically, Gram-negative bacterial resistance genes often express efflux pumps, which utilize active transport to expel antimicrobials from the bacterial cell.[10]

Gram-negative bacteria exhibit increased intrinsic resistance to antibiotics compared to their Gram-positive counterparts due to the presence of an outer lipopolysaccharide layer.[10] This outer membrane consists of glycolipids within the outer leaflet of the bacterium and intrinsically limits the influx of hydrophilic antibiotic solutes into the cell, contributing to their pathogenicity and multidrug resistance.[11] Clinically important multidrug-resistant Gram-negative organisms include *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter species*.[8]

Additionally, the administration of antibiotics to animals in agricultural settings and the fecal excretion of antibiotics into the environment contribute to environmental contamination. This issue is further compounded by the use of manure as fertilizer.[12] The presence of antibiotics in the environment, in combination with resistance acquired through horizontal gene transfer, promotes the further development of antimicrobial resistance.

Waterways and reservoirs within metropolitan areas are at increased risk of harboring multidrug-resistant bacteria due to their proximity to urban hospitals and runoff from agricultural sites.[3,4] The Eagle Creek Reservoir in Indianapolis, Indiana, is part of the White River watershed and receives runoff from surrounding urban and agricultural areas. Due to the accumulation of organisms from regions with increased antibiotic usage, Eagle Creek provides insight into potential antimicrobial resistance patterns that may emerge.

Materials and Methods

To determine the specific antibiotic resistances within Gram-negative bacteria, one liter of water was collected from four different sites within Eagle Creek Reservoir, totaling 4 L of water. These samples were collected in the summer and were stored between 2 °C to 8 °C. The time between collection and storage of these samples was 1 week. These water samples were then vacuum filtered through increasingly fine Millipore® filters, from a paper filter, to 10 µm, 6 µm, 2.5 µm, 1.2 µm, and 0.6 µm, with the final filtration pore size at 0.45 µm. Filtering was completed to isolate bacteria from debris, fungi, and other microorganisms. These filters were then plated on MacConkey agar and incubated at 37 °C for 24 hours. To isolate Gram-negative bacteria, single colonies from each filter paper were serially plated on additional MacConkey agar plates and incubated at 37 °C for 24 hours

until pure culture was obtained. Single colonies were transferred from MacConkey agar to nutrient agar and incubated at 37 °C for 24 hours. Following growth on nutrient agar, a single colony was restreaked on Mueller-Hinton (MH) agar and incubated at 37 °C for 24 hours. The next day, 3-5 isolated colonies from the overnight MH agar plates were inoculated into 5 mL Mueller-Hinton broth and incubated in a shaker at 37 °C, 200 rpm, for three to four hours. After 3-4 hours of growth, optical density reading of each isolate was taken and they were diluted using MH broth to an optical density equivalent to a 0.5 McFarland standard (OD ≈ 0.08–0.13) as measured by spectrophotometry. The standardized inoculum was swabbed evenly across the surface of Mueller-Hinton agar plates to produce a uniform bacterial lawn. Antibiotic disks were placed on the agar surface using sterile forceps with adequate spacing to prevent overlapping zones of inhibition. Plates were incubated at 37 °C for approximately 18–24 hours. Following incubation, the zones of inhibition surrounding each antibiotic disk were measured and interpreted according to Clinical and Laboratory Standards Institute (CLSI) susceptibility guidelines. The antibiotics tested included commonly used agents in healthcare and agricultural settings: amoxicillin/clavulanic acid (20/10 µg), ampicillin (10 µg), cephalothin (30 µg), cefoxitin (30 µg), ceftiofur (30 µg), ceftriaxone (30 µg), imipenem (10 µg), sulfisoxazole (250 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg), amikacin (30 µg), gentamicin (10 µg), kanamycin (30 µg), streptomycin (10 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), chloramphenicol (30 µg), and tetracycline (30 µg). As a control, Kirby Bauer assay was also performed on an *E. coli* reference strain, ATCC 25922, a CLSI control strain which has a known susceptibility profile against the antibiotics being tested.

Results

Kirby-Bauer disk diffusion assay of all 72 isolates revealed that each displayed resistance to at least one antibiotic, with 69 isolates (95%) demonstrating multidrug resistance to two or more antibiotics.

Resistance profile analysis of the 72 isolates revealed a mean resistance count of 5.5 antibiotics per isolate, ranging from 0 to 12. The most prevalent resistance was observed among the beta-lactam antibiotics, with 95.8% of isolates resistant to ampicillin and 79.2% resistant to amoxicillin-clavulanate. Sulfisoxazole (65.3%) and cephalothin (52.8%) were the next most frequently observed resistances. Within the carbapenem class, 51.4% of isolates displayed imipenem resistance. Among the aminoglycosides, streptomycin demonstrated the highest resistance rate at 30.6%, while gentamicin had the lowest overall resistance rate across all antibiotics tested at 5.6%.

Analysis of co-resistance identified ampicillin and amoxicillin-clavulanate as the most frequently co-occurring resistance pair, present together in 77.8% of isolates. The next most common co-resistance pairs were ampicillin with sulfisoxazole (63.9%) and amoxicillin-clavulanate with sulfisoxazole (55.6%). Ampicillin and imipenem co-resistance was observed in 51.4% of isolates, and ampicillin and cephalothin co-resistance in 50.0%.

Resistance profile clustering identified three recurring multidrug-resistant phenotypes among the isolates. The most common profile, shared by six isolates, comprised co-resistance to amoxicillin-clavulanate, ampicillin, imipenem, and sulfisoxazole. Two distinct profiles tied for the next most frequent, each observed in four isolates: one consisting of co-resistance to amoxicillin-clavulanate, ampicillin, cephalothin, cefoxitin, and tetracycline, and the other consisting of co-resistance to amoxicillin-clavulanate, ampicillin, cephalothin, cefoxitin, ceftiofur, and ceftriaxone. Three isolates shared a five-antibiotic profile of amoxicillin-clavulanate, ampicillin, cephalothin, imipenem, and sulfisoxazole, and two isolates were monoresistant to ampicillin alone. At the opposite extreme, two isolates demonstrated resistance to 13 antibiotics, with susceptibility retained only to amikacin, ciprofloxacin, gentamicin, and imipenem.

Discussion

The results of this study demonstrate a high prevalence of antibiotic resistance among gram-negative bacteria isolated from Eagle Creek, with all isolates displaying resistance to a least one antibiotic and 95% being multidrug

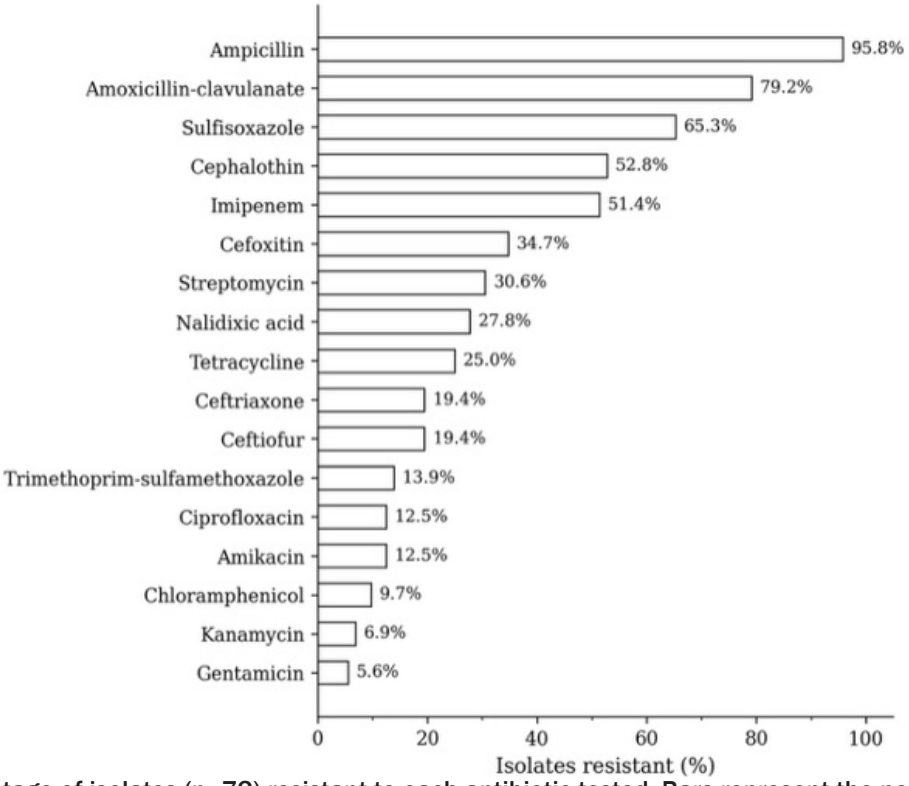

Figure 1. Percentage of isolates (n=72) resistant to each antibiotic tested. Bars represent the percentage of isolates classified as resistant (R) by Kirby-Bauer disk diffusion assay.

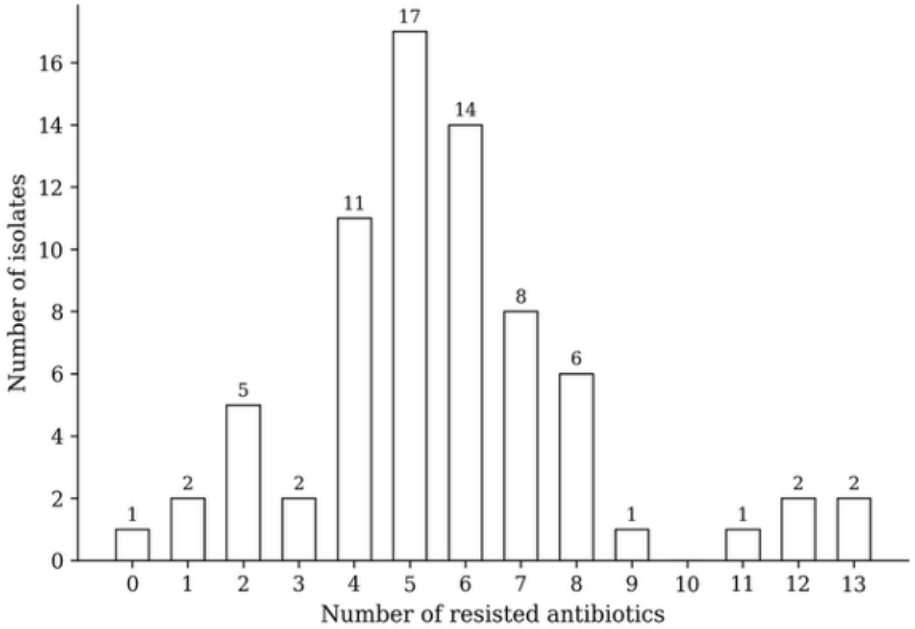

Figure 2. Distribution of antibiotic restance counts per isolate (n=72). Each bar represents the number of isolates (y-axis) resistant to a given number of antibiotics (x-axis). Numbers above bars indeciate isolate counts.

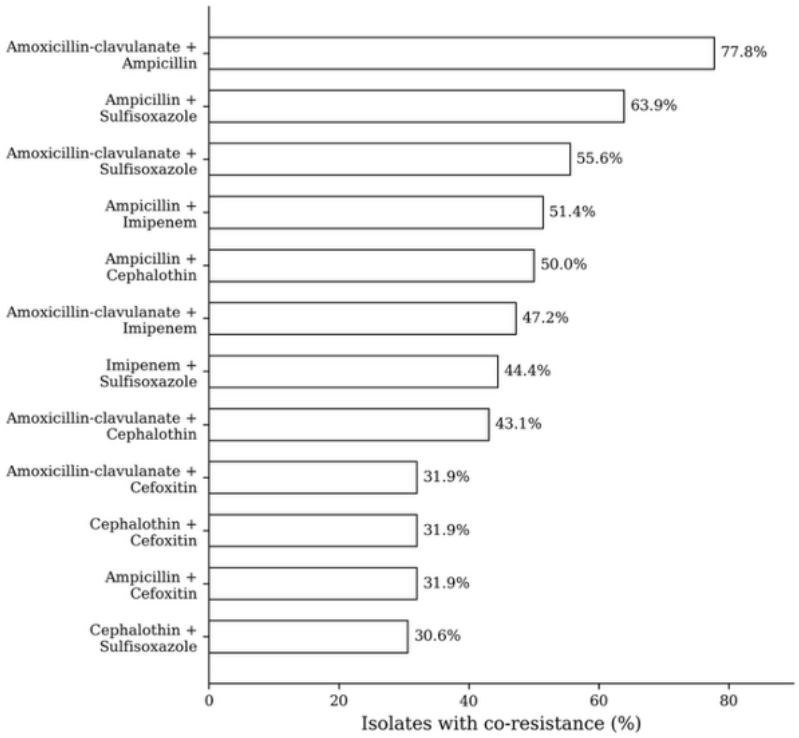

Figure 3. Top 12 most frequent antibiotic co-resistance pairs among isolates (n=72). Bars represent the percentage of isolates displaying resistance to both antibiotics in each pair.

resistant. These findings are consistent with previous studies that have documented that urban surface waters are reservoirs for antibiotic-resistant bacteria. Similar studies situated in urban waterways adjacent to hospital drainage and rural waterways receiving agricultural runoff have reported comparable rates of multidrug resistance among Gram-negative isolates.[3,4] Eagle Creek Reservoir's unique position within a watershed receiving both agricultural and urban drainage, and the corresponding high rate of antibiotic resistance, reinforces the possible pressure human expansion and development have on the development of antimicrobial resistance.

The predominance of penicillin resistance aligns with global surveillance data identifying penicillins as among the most compromised antibiotic classes in environmental settings.[13] Resistance to ampicillin (95.8%) and amoxicillin-clavulanate (79.2%) was observed at rates consistent with or exceeding those reported in global surveillance among Gram-negative bacteria.[13] Beta-lactam resistance in Gram-negative environmental bacteria is most commonly mediated by plasmid-encoded beta-lactamases, which are readily transferred between bacterial species.[9] Additionally, the high rate of co-resistance between these two agents supports possible plasmid-mediated beta-lactamase expression rather than species-specific chromosomal mutations. Given the high frequency of prescription of beta-lactam antibiotics, ongoing selection pressure in urban runoff entering the White River watershed may sustain and amplify resistance within the local microbiome.[13]

One clinically notable finding is the resistance to imipenem observed in 51.4% of isolates. Carbapenems are utilized in the treatment of multidrug-resistant gram-negative organisms, typically after the exhaustion of other therapies.[14] Increasing resistance to carbapenems severely limits clinicians' ability to treat severe, multidrug-resistant infections. Carbapenem resistance in gram-negative bacteria can arise through different pathways, including carbapenemase enzyme production, loss or downregulation of outer membrane porins, and upregulation of efflux pump systems.[15] The specific resistance

mechanisms are not characterized in the present study, however, the frequency of imipenem resistance observed is higher than the rates reported in comparable reservoir studies.[3,4] This warrants continued characterization and monitoring of the Eagle Creek watershed to inform strategies aimed at limiting transmission of carbapenem resistance to pathogenic bacteria.

The high rate of sulfisoxazole resistance (65.3%) and the frequent co-resistance associations identified between sulfisoxazole and both ampicillin (63.9%) and amoxicillin-clavulanate (55.6%) suggest the possibility of mobile genetic elements capable of co-selecting for resistance across multiple antibiotic classes.[5,7] This is evidenced by the resistance to a range of different antibiotic classes rather than narrow, species-specific chromosomal mutations for resistance to only one class of antibiotics.

Aminoglycoside antibiotics exhibited the lowest resistance rates of all classes tested, with gentamicin showing relatively low resistance rate at 5.6%. No isolate demonstrated resistance to all aminoglycosides tested, distinguishing this class from others within the panel. This relative preservation of aminoglycoside susceptibility may reflect reduced selective pressure compared to beta-lactams in the local environment due to less frequent prescription in the United States.[16] However, streptomycin resistance was 30.6%, the highest among the aminoglycosides, and streptomycin is largely only used in veterinary medicine.[4] This further suggests that decreased rates of prescription and administration in veterinary medicine may lead to a reduction in environmental bacterial resistance.

The public health implications of these findings may be relevant to Indianapolis. Eagle Creek Reservoir contributes to the White River watershed, which serves as a source for municipal water distribution in the city.[17] Although conventional water treatment processes reduce viable bacterial loads, they do not reliably eliminate extracellular resistance genes or DNA-containing particles.[18] Even with proper decontamination of water, bacteria are able to transfer resistance genes that can assist with colonization within water infrastructure. The presence of multidrug-resistant Gram-negative bacteria in source water, particularly carbapenem-resistant bacteria, presents a possible exposure to clinically relevant resistance phenotypes. Additionally, individuals with recreational contact with Eagle Creek, including swimmers, fishers, and kayakers, face additional risk of direct exposure. Continued monitoring and surveillance of the Eagle Creek antimicrobial resistance profile can assist local health authorities in anticipating and responding to emerging resistance.

Limitations of this study should be considered when interpreting these results. Bacterial isolates were not identified at the species level, which precludes the ability to correlate resistance profiles with known intrinsic resistance of bacterial species. The genetic basis of resistance was not characterized in this study; therefore, the specific resistance genes remain unconfirmed. This study was also limited to aerobically cultured Gram-negative organisms, which may underrepresent the true diversity of bacteria within Eagle Creek. Because Indianapolis experiences substantial seasonal variation, resistance patterns may vary by season and were not assessed in this study. Future work planned to supplement this study includes incorporating 16S rRNA-based sequencing for species identification and characterization of resistance genes and plasmids. Additionally, longitudinal sampling across seasons could be performed to identify patterns in resistance throughout the year.

Conclusion

Eagle Creek Reservoir harbors a diverse community of antibiotic-resistant gram-negative bacteria, whose resistance profiles may reflect the combination of receiving urban hospital and rural agricultural runoff. The high rates of resistance to ampicillin and amoxicillin-clavulanate are consistent with patterns reported in previous literature and may reflect selective pressures from nosocomial and agricultural antibiotic use in aquatic environments. Most notably, the detection of imipenem resistance in 51.4% of isolates has clinical relevance, as carbapenem resistance in a source of utility and drinking water would pose a risk for transfer of resistance to last-resort antibiotics.

The predominance of multidrug resistant phenotypes, the clustering of co-resistance profiles, and the absence of any antibiotic class to which

all isolates are susceptible indicate that Eagle Creek may be an environment that fosters multidrug resistance. This highlights the need for surveillance strategies and continued monitoring of the antibiotic resistance profile to protect the population and livestock from clinically significant infections.

Future studies that include bacterial species identification, whole-genome sequencing, and plasmid characterization will determine the specific genetic contributions to resistance in Eagle Creek isolates. Longitudinal sampling across seasons and additional collection sites within the Eagle Creek watershed will further clarify and complete its resistance profile. Continued surveillance and monitoring of the Eagle Creek watershed may help inform agricultural and public health efforts to monitor and mitigate antimicrobial resistance.

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