Proceedings of College of STEM Symposium
PROC(05ACSS2026)003
Clayton State University
03/2026

Characterization and Antimicrobial Resistance Profiling of Freshwater Bacterial Isolates from Reynolds Nature Preserve

Anistasia Keoseng, Maria Martinez-Villavicencio, Jovaughn Redley, Clint Edmunds, PhD, Paul D. Melvin, PhD, and Vivian Padín-Irizarry, PhD*
College of STEM, School of Sciences, Clayton State University, Morrow, GA 30260 USA

Abstract. Freshwater environments are recognized reservoirs of environmentally acquired antimicrobial resistance (AMR), presenting risks to public and ecosystem health. In this study, bacterial isolates were collected from Reynolds Nature Preserve, a local freshwater site in Clayton County, Georgia, and characterized using standard microbiological methods including Gram staining, biochemical assays, and Kirby-Bauer disk diffusion susceptibility testing. All three isolates (C1, C2, and D2) were identified as Gram-negative, non-motile, citrate-positive rods consistent with environmental enteric bacteria, with preliminary profiles suggestive of *Klebsiella* spp. and *Escherichia* spp. Antibiotic susceptibility testing indicated broad susceptibility across most clinical antibiotics, with the exception of intrinsic erythromycin resistance. Water chemistry analysis revealed soft, slightly acidic freshwater (pH 6.3) with low ionic strength and total dissolved solids, and the presence of total coliforms but no detectable *E. coli* or fecal coliforms. Future work will include 16S rRNA gene sequencing for definitive species identification and molecular screening for AMR gene determinants. These findings contribute to an emerging understanding of environmental AMR at the community level and underscore the value of integrating undergraduate researchers in active public-health-relevant science.

1 Introduction

Freshwater ecosystems harbor diverse microbial communities that play essential roles in nutrient cycling and ecological balance. However, these environments also serve as interfaces for the accumulation and dissemination of antimicrobial resistance (AMR) genes and resistant organisms [1]. Environmental bacteria exposed to anthropogenic inputs, including agricultural runoff, wastewater effluent, and pharmaceutical residues, can acquire, maintain, and transfer resistance determinants to clinically relevant pathogens through horizontal gene transfer. The identification and characterization of AMR-carrying organisms in natural freshwater systems is therefore critical for understanding the ecology of resistance and anticipating emerging public health threats.

Reynolds Nature Preserve is a local freshwater site situated in Clayton County, Georgia, providing a representative setting to evaluate microbial diversity and antibiotic resistance in a peri-urban natural waterway. Despite its ecological value as a community green space, the preserve is adjacent to suburban development, making it a plausible receptor for non-point-source bacterial contamination. To date, no systematic characterization of the preserve's freshwater microbiome or AMR landscape has been conducted.

The goals of this study were threefold: (1) to isolate and phenotypically characterize culturable Gram-negative bacteria from freshwater samples collected at Reynolds Nature Preserve; (2) to profile antibiotic susceptibility patterns using standardized Kirby-Bauer disk diffusion methodology per Clinical and Laboratory Standards Institute (CLSI) guidelines [2]; and (3) to assess basic water quality parameters at sample collection sites. Collectively, these data lay the groundwork for subsequent 16S rRNA gene sequencing and molecular AMR gene detection, with the broader aim of establishing a longitudinal dataset of environmental AMR at this site.

2 Materials and Methods

2.1 Site Description and Sample Collection

Water samples were collected from two distinct sites (C and D) at Reynolds Nature Preserve, Clayton County, Georgia, using sterile 50-mL conical tubes. Samples were processed within 24 hours of collection. A distilled water sample and a sink water sample were included as negative and positive controls, respectively, for water chemistry and microbiological testing.

* VivianPadin-Irizarry@clayton.edu

2.2 Bacterial Isolation and Cultivation

All water samples were plated onto Nutrient Agar (NA) and MacConkey Agar using aseptic technique and the streak-plate method to obtain pure isolates. Plates were incubated at 37°C for 24 hours. Individual morphologically distinct colonies were selected and subcultured to purity. Three isolates, designated C1, C2 (from Site C), and D2 (from Site D), were selected for further characterization. Fresh cultures were prepared within 24 hours prior to each biochemical assay.

2.3 Phenotypic and Biochemical Characterization

Gram staining was performed according to standard protocol to determine cell wall morphology and Gram reaction. Motility was assessed in soft agar (0.3% agar). A full panel of biochemical tests was conducted including: Triple Sugar Iron (TSI) agar for carbohydrate fermentation and hydrogen sulfide production; Simmons Citrate agar; urease broth; Eosin-Methylene Blue (EMB) agar; and MacConkey agar. An *Escherichia coli* ATCC strain was included as a quality-control reference organism in all biochemical panels.

2.4 Antibiotic Susceptibility Testing

Antibiotic susceptibility was determined using the Kirby-Bauer disk diffusion method on Mueller-Hinton Agar (MHA) according to CLSI M02 guidelines [2]. A 0.5 McFarland turbidity standard was used to normalize inoculum density. Antibiotic disks tested included Ampicillin (10 µg), Amoxicillin/Clavulanate (30 µg), Cefazolin (30 µg), Ciprofloxacin (5 µg), and Trimethoprim/Sulfamethoxazole (25 µg). Erythromycin (15 µg) was included as a control for intrinsic resistance in Gram-negative organisms. Zones of inhibition were measured in millimeters after 18–24 hours of incubation at 35°C and interpreted as Susceptible (S), Intermediate (I), or Resistant (R) according to published CLSI breakpoints.

2.5 Water Chemistry and Microbiological Analysis

Water chemistry was assessed using the Verify™ Complete Water Test Kit strip system for pH, nitrate, nitrite, total chlorine, iron, manganese, and mercury. Total dissolved solids (TDS) were measured using a calibrated digital TDS meter. Microbiological water quality was evaluated using the Verify™ Bacteria Test for Water, which detects the presence of total coliforms and *E. coli* / fecal coliforms via enzyme substrate methodology.

3 Results

3.1 Biochemical Characterization of Isolates

All three isolates (C1, C2, and D2) were confirmed as Gram-negative rods, displaying consistent pink-staining morphology under light microscopy. Growth was observed on both MacConkey and EMB agars, indicating tolerance of bile salts and selective media consistent with the Enterobacteriaceae family. All isolates were non-motile in soft agar and uniformly negative in urease testing. Citrate utilization was positive for all three isolates, distinguishing them from the *E. coli* control (citrate-negative). TSI results revealed variable carbohydrate fermentation patterns: isolates C1 and D2 exhibited alkaline slants with acidic butts, whereas C2 produced an acid/acid pattern consistent with glucose and lactose fermentation. No hydrogen sulfide production was detected in any isolate. Based on the aggregate biochemical profile, specifically citrate positivity, non-motility, absence of urease, and differential TSI patterns, preliminary identification suggests the presence of *Klebsiella* spp. (C1, D2) and *Escherichia* spp. (C2) (Table 1).

3.2 Antibiotic Susceptibility Profiles

Kirby-Bauer disk diffusion results indicated that all three isolates were broadly susceptible (S) to the five clinical antibiotics tested: Ampicillin, Amoxicillin/Clavulanate, Cefazolin, Ciprofloxacin, and Trimethoprim/Sulfamethoxazole (Table 2). Zone of inhibition measurements ranged from 16–30 mm across isolates and antibiotic classes, with Ciprofloxacin producing the largest zones consistent with its broad-spectrum activity. As expected, all isolates demonstrated resistance (R) to Erythromycin, reflecting the intrinsic resistance of Gram-negative bacteria to this macrolide due to outer membrane impermeability. Representative photographs of the disk diffusion assay are shown in Figure 1.

Table 1. Biochemical characterization results for isolates C1, C2, D2, and E. coli reference control.

Test	C1	C2	D2	E. coli (control)
Gram Stain	Negative	Negative	Negative	Negative
Motility	Non-motile	Non-motile	Non-motile	Motile
Citrate	Positive	Positive	Positive	Negative
Urease	Negative	Negative	Negative	Negative
TSI (Slant/Butt)	Alk/Acid	Acid/Acid	Alk/Acid	Acid/Acid
H2S Production	Negative	Negative	Negative	Negative
Gas Production	Positive	Positive	Positive	Positive
EMB Growth	Positive	Positive	Positive	Positive
MacConkey Growth	Positive	Positive	Positive	Positive

Table 2. Antibiotic resistance profiles and zone of inhibition measurements (mm) for isolates C1, C2, and D2, with E. coli reference control. *S = Susceptible; R = Resistant (per CLSI M02 breakpoints). Erythromycin included as intrinsic resistance control for Gram-negative organisms. Zone diameters reported as mean of triplicate measurements.*

Antibiotic (Disk Conc.)	C1 (mm)	C2 (mm)	D2 (mm)	Interpretation (S/I/R)
Ampicillin (10 µg)	18	17	16	S / S / S
Amoxicillin/Clavulanate (30 µg)	22	21	20	S / S / S
Cefazolin (30 µg)	24	23	22	S / S / S
Ciprofloxacin (5 µg)	30	29	28	S / S / S
Trimethoprim/Sulfa (25 µg)	26	24	25	S / S / S
Erythromycin (15 µg) (control)	0	0	0	R / R / R (intrinsic)

3.3 Water Chemistry and Microbiological Quality

Both freshwater sample sites (C and D) exhibited consistent physicochemical profiles: slightly acidic pH (6.3), low total dissolved solids, and non-detectable levels of nitrate, nitrite, total chlorine, iron, manganese, and mercury (Table 3). This profile is characteristic of soft, low-ionic-strength freshwater with minimal anthropogenic nutrient loading. Microbiological analysis detected total coliforms in both Sample C and Sample D, indicating the presence of environmental bacteria; however, *E. coli* and fecal coliforms were not detected in either sample. Control samples (distilled water and sink water) showed expected results, supporting assay validity.

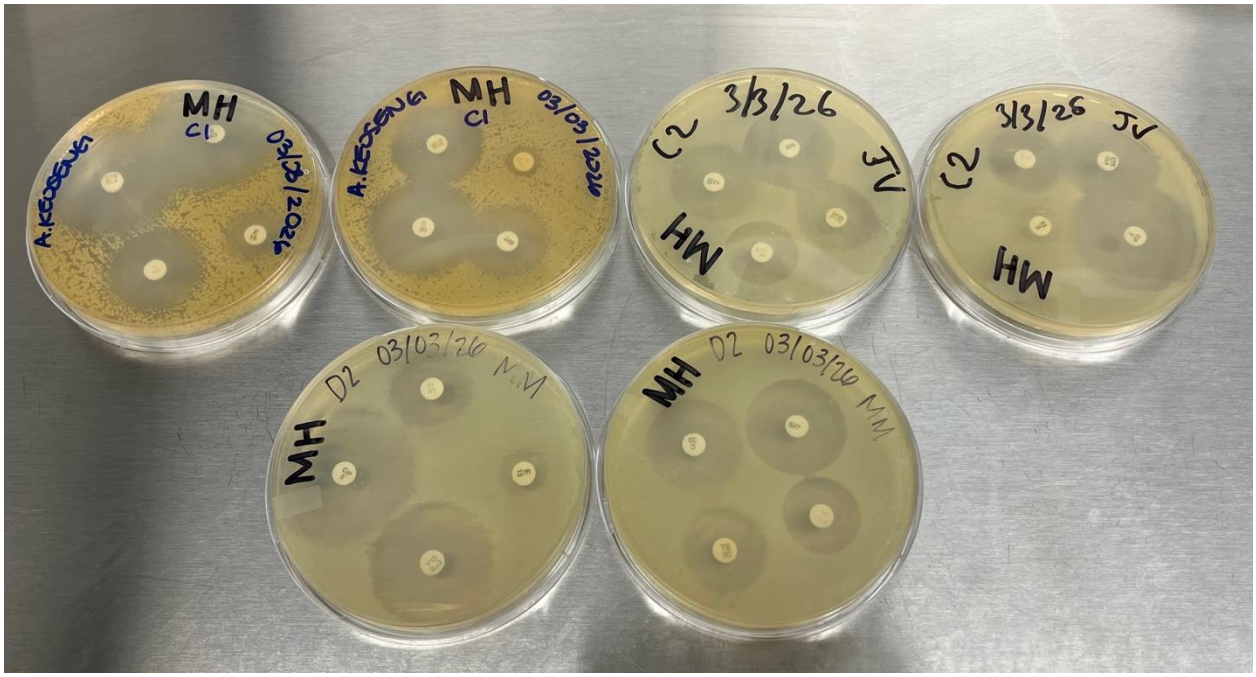

Fig. 1. Representative Kirby-Bauer disk diffusion assay plates for isolates C1 (top left), C2 (top right center), and D2 (bottom).

Table 3. Water chemistry and microbiological parameters for sample sites C, D, and controls.

Parameter	Sample C	Sample D	Distilled H_2O (ctrl)	Sink Water (control)
pH	6.3	6.3	7.0	7.2
TDS† (mg/L)	Low	Low	~0	Variable
Nitrate	Not detected	Not detected	Not detected	Not detected
Nitrite	Not detected	Not detected	Not detected	Not detected
Total Chlorine	Not detected	Not detected	Not detected	Detected
Iron	Not detected	Not detected	Not detected	Not detected
Manganese	Not detected	Not detected	Not detected	Not detected
Mercury	Not detected	Not detected	Not detected	Not detected
Total Coliforms	Detected	Detected	Not detected	Not detected
E. coli / Fecal coliforms	Not detected	Not detected	Not detected	Not detected

4 Discussion

The isolation of Gram-negative, citrate-positive, non-motile rod-shaped bacteria from both collection sites at Reynolds Nature Preserve is consistent with the known distribution of Enterobacteriaceae in freshwater environments. The preliminary biochemical profiles for isolates C1 and D2 align most closely with *Klebsiella* spp., which are ubiquitous in environmental water and soil and are increasingly recognized as opportunistic pathogens capable of harboring extended-spectrum beta-lactamases (ESBLs) and carbapenemases [3]. Isolate C2's acid/acid TSI pattern and citrate positivity warrant further distinction between *Klebsiella* and fermentative *Enterobacter* species; definitive assignment will require molecular confirmation.

The observed susceptibility to frontline clinical antibiotics (ampicillin, ciprofloxacin, trimethoprim/sulfamethoxazole, and first-generation cephalosporins) is notable and may reflect the preserve's relatively low-impact land use. The absence of fecal coliforms and low nutrient indicators further suggests limited sewage or agricultural effluent input. Nevertheless, the detection of total coliforms in both field samples, absent in the distilled water control, confirms microbial contamination of unknown origin, warranting continued monitoring.

† *TDS = Total Dissolved Solids. All parameters assessed using Verify™ Complete Water Test Kit and Verify™ Bacteria Test for Water. Distilled water and sink water are included as negative and positive controls, respectively.*

The intrinsic erythromycin resistance observed across all isolates is an expected phenotype for Gram-negative organisms, resulting from the impermeability of the outer membrane to macrolide antibiotics rather than acquired resistance mechanisms. Its inclusion as a control confirmed the validity of the disk diffusion methodology and the ability of isolates to produce interpretable inhibition zone patterns.

A limitation of the current study is the reliance on phenotypic biochemical identification, which lacks the resolution of modern molecular methods. Biochemical panels can misclassify closely related Enterobacteriaceae species, and the true diversity of culturable and non-culturable organisms at the site cannot be assessed by culture-based methods alone. Additionally, the current antibiotic panel does not include carbapenems or higher-generation cephalosporins, which would be necessary to detect more clinically significant resistance phenotypes.

4.1 Future Directions

The next phase of this project will involve DNA extraction from isolates C1, C2, and D2 followed by 16S rRNA gene amplification and sequencing (Sanger or amplicon-based), enabling species-level identification with significantly greater taxonomic precision. Concurrently, whole-community 16S rRNA amplicon sequencing from environmental DNA (eDNA) will be pursued to capture the full diversity of the freshwater microbiome at Reynolds Nature Preserve beyond the limitations of culture-based approaches.

5 Conclusions

This study provides a phenotypic characterization of culturable Gram-negative bacterial isolates from Reynolds Nature Preserve and describes their baseline antibiotic susceptibility profiles. The isolates, preliminarily identified as Klebsiella spp. and Escherichia spp., exhibit broad susceptibility to clinically relevant antibiotics, suggesting that resistance pressure at this site is currently low. The detection of total coliforms without fecal indicators points to environmental contamination not associated with sewage, consistent with stormwater or soil runoff. These findings establish a foundation for ongoing molecular and ecological investigation and underscore the importance of undergraduate-led environmental surveillance as both a scientific and pedagogical enterprise.

Acknowledgments

The authors thank Dr. Boey Khem Tan for laboratory support and technical guidance throughout this project.

References

1. P. S. Cabral, Water Microbiology. Bacterial pathogens and water, *Int. J. Environ. Res. Public Health*, **7**, 3657–3703 (2010).
2. Clinical and Laboratory Standards Institute, CLSI Standard M02, **14th ed**. (2023)
3. Z. A. Khan, M.F. Siddiqui, S. Park, Current and Emerging Methods of Antibiotic Susceptibility Testing, *Diagnostics (Basel)*, **9**, 49 (2019).