January 2020

Antimicrobial Resistance And Molecular Epidemiology Of Escherichia Coli Isolated From Urban And Rural River Systems

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2020

Master of Public Health Candidate

Yale School of Public Health

Epidemiology of Microbial Diseases

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ABSTRACT

Antimicrobial resistance (AMR) is an emerging public health issue that threatens the efficacy of antibiotic treatment for bacterial infections and human health. Sources of antimicrobial resistance genes (ARGs) in the environment include wastewater treatment plants and animal feeding operations that discharge waste into waterways, such as rivers and streams. This retrospective descriptive study describes the presence of AMR, and specific ARGs in *Escherichia coli* isolates from two distinct watersheds, rural and urban, with the use of antimicrobial susceptibility testing, whole-genome sequencing (WGS) to detect ARGs, and multi-locus sequence typing. Antimicrobial susceptibility testing was performed for 143 *E. coli* isolates, 73 originating from a rural watershed and 70 originating from an urban watershed. *E. coli* isolates from the rural watershed had a significantly higher prevalence of phenotypic non-susceptibility and ARGs for tetracycline (21.9% vs. 2.9%, $p < 0.01$) when compared to urban watershed isolates. Based on phenotypic-susceptibility testing, WGS data of 68 isolates were annotated for ARGs. These data were used for the prediction of antimicrobial susceptibility, demonstrating high accuracy for the prediction of non-susceptibility for tetracycline, trimethoprim-sulfamethoxazole, and cephalosporins. WGS multi-locus sequence typing (MLST) yielded 47 sequence types, dominated by ST58 (n=6), ST10 (n=5), and ST155 (n=4). Waterways are important reservoirs and disseminators of antimicrobial-resistant bacteria (ARB) and ARGs. The evaluation and monitoring of AMR and ARGs in aquatic environments will lead to improved health through better prevention and control of *E. coli* infections.

ACKNOWLEDGMENTS

The author of this project acknowledges Dr. Melinda Pettigrew, Professor of Epidemiology (Microbial Diseases), and Dr. Windy Tanner, Associate Research Scientist in Epidemiology (Microbial Diseases), for the support and guidance throughout the formation of this thesis. Additionally, the author acknowledges Dr. Windy Tanner and colleagues, Dr. James VanDerslice and Dr. William Brazelton, from the University of Utah for the collection of isolates and initial reports, integral to the formation of this thesis. Finally, the author acknowledges the Yale School of Public Health community, faculty, and students for continued academic support throughout the formation of this thesis.

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Table 1. Antimicrobial Non-Susceptibility of *E. coli* isolates from a rural and urban watershed

Antimicrobial Agent	Number of Non-Susceptible Isolates (%)			p-value
	Total (n = 143)	Rural (n = 73)	Urban (n = 70)	
Aminoglycoside				
Gentamicin	0 (0)	0 (0)	0 (0)	-
Beta-lactam				
Ampicillin	33 (23.1)	19 (26.0)	14 (20.0)	0.43
Amoxicillin-clavulanic acid	20 (14.0)	12 (16.4)	8 (11.4)	0.47
Aztreonam*	2 (1.4)	1 (1.4)	1 (1.4)	1.00
Cefazolin	118 (82.5)	63 (86.3)	55 (78.6)	0.27
Cefazolin (Uncomplicated UTIs)	3 (2.1)	1 (1.4)	2 (2.9)	0.61
Cefepime	2 (1.4)	1 (1.4)	1 (1.4)	1.00
Cefotaxime	3 (2.1)	1 (1.4)	2 (2.9)	0.61
Cefoxitin	8 (5.6)	2 (2.7)	6 (8.6)	0.16
Ceftazidime	2 (1.4)	0 (0)	2 (2.9)	0.24
Ceftriaxone	4 (2.8)	3 (4.1)	1 (1.4)	0.62
Meropenem*	2 (1.4)	1 (1.4)	1 (1.4)	1.00
Quinolone				
Ciprofloxacin*	1 (0.7)	0 (0)	1 (1.4)	0.49
Tetracycline				
Tetracycline	18 (12.6)	16 (21.9)	2 (2.9)	< 0.01
Trimethoprim-sulfonamide				
Trimethoprim-sulfamethoxazole*	7 (4.9)	6 (8.2)	1 (1.4)	0.12

* 142 of 143 isolates were tested for antimicrobial susceptibility

The Kirby-Bauer method was used to determine resistance profiles for gentamicin, ceftazidime, cefotaxime, aztreonam, ceftriaxone, cefazolin, cefoxitin, cefepime, ampicillin, amoxicillin with clavulanic acid, meropenem, ciprofloxacin, tetracycline, and sulfamethoxazole-trimethoprim. Interpretation of the results of the resistance profiles was performed using the 2018 CLSI criteria [22]. Rural and urban values were evaluated with Fisher's exact test. A two-tailed p-value of <0.05 was considered statistically significant.

Table 2. Antimicrobial resistance genes detected from the Structured Antimicrobial Resistance Genes (SARG) v2.0 database present in a subset of *E. coli* isolates from a rural and urban watershed

Antimicrobial Class	Number of Isolates with one or more antimicrobial resistance gene (%)		
	Total (n = 68)	Rural (n = 37)	Urban (n = 31)
Aminoglycoside	0 (0)	0 (0)	0 (0)
Bacitracin	67 (98.5)	37 (100.0)	30 (96.8)
Beta-lactam	8 (11.8)	5 (13.5)	3 (9.7)
Bleomycin	0 (0)	0 (0)	0 (0)
Carbomycin	0 (0)	0 (0)	0 (0)
Chloramphenicol	2 (2.9)	2 (5.4)	0 (0)
Fosfomycin	0 (0)	0 (0)	0 (0)
Fosmidomycin	1 (1.5)	1 (2.7)	0 (0)
Fusaric-acid	0 (0)	0 (0)	0 (0)
Fusidic-acid	0 (0)	0 (0)	0 (0)
Kasugamycin	0 (0)	0 (0)	0 (0)
Macrolide-lincosamide-streptogramin	1 (1.5)	1 (2.7)	0 (0)
Multidrug	68 (100.0)	37 (100.0)	31 (100.0)
Polymyxin	66 (97.1)	36 (97.3)	30 (96.8)
Puromycin	0 (0)	0 (0)	0 (0)
Quinolone	0 (0)	0 (0)	0 (0)
Rifamycin	0 (0)	0 (0)	0 (0)
Spectinomycin	0 (0)	0 (0)	0 (0)
Sulfonamide	7 (10.3)	6 (16.2)	1 (3.2)
Tetracenomycin	0 (0)	0 (0)	0 (0)
Tetracycline	13 (19.1)	12 (32.4)	1 (3.2)
Trimethoprim	3 (4.4)	3 (8.1)	0 (0)
Unclassified	1 (1.5)	0 (0)	1 (3.2)
Vancomycin	0 (0)	0 (0)	0 (0)

Whole-genome sequences of 68 *E. coli* isolates were annotated for ARGs that are associated with antimicrobial agent classes from the SARG v2.0 database [27].

Table 3. Performance of genotypic resistance data for the prediction of phenotypic antimicrobial non-susceptibility in in *E. coli* isolates

Antimicrobial Resistance Gene	Antibiotic	Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)	Accuracy (%)
tetA	Tetracycline	100.0%	79.7%	23.5%	100.0%	80.9%
tetC		100.0%	87.9%	58.8%	100.0%	89.7%
tetD		100.0%	78.5%	17.6%	100.0%	79.4%
tetR		100.0%	79.7%	23.5%	100.0%	80.9%
dfrA5	Trimethoprim-sulfamethoxazole	100.0%	95.4%	50.0%	100.0%	95.6%
sul2		85.7%	100.0%	100.0%	98.4%	98.5%
CMY-2	Cefazolin	100.0%	16.4%	1.8%	100.0%	17.6%
	Cefazolin (Uncomplicated UTI)	100.0%	98.5%	50.0%	100.0%	98.5%
	Cefoxitin	100.0%	91.0%	14.3%	100.0%	91.2%
	Cefotaxime	100.0%	98.5%	50.0%	100.0%	98.5%
	Ceftazidime	100.0%	100.0%	100.0%	100.0%	100.0%
	Ceftriaxone	100.0%	98.5%	50.0%	100.0%	98.5%
	Cefepime	NA	98.5%	NA	98.5%	97.1%
	Ampicillin	100.0%	67.2%	4.3%	100.0%	67.6%
	Amoxicillin-clavulanic acid	100.0%	77.6%	6.3%	100.0%	77.9%
TEM-1	Cefazolin	100.0%	18.0%	12.3%	100.0%	26.5%
	Cefazolin (Uncomplicated UTI)	NA	96.7%	NA	89.4%	86.8%
	Cefoxitin	NA	88.5%	NA	88.5%	79.4%
	Cefotaxime	NA	96.7%	NA	89.4%	86.8%
	Ceftazidime	NA	98.4%	NA	89.6%	88.2%
	Ceftriaxone	NA	96.7%	NA	89.4%	86.8%
	Cefepime	NA	98.4%	NA	89.6%	88.2%
	Ampicillin	100.0%	73.8%	30.4%	100.0%	76.5%
	Amoxicillin-clavulanic acid	57.1%	80.3%	25.0%	94.2%	77.9%

Susceptibility and non-susceptibility predictions were performed for 68 *E. coli* isolates collected from a rural and urban watershed using antimicrobial resistance genes in the Structured Antimicrobial Resistance Genes (SARG) v2.0 database [27]. These antimicrobial resistance genes were used to predict the conference of non-susceptibility for antibiotics associated with the gene.

Table 4. Multi-locus sequence types of 68 *E. coli* Isolates from environmental water samples

E. coli Isolate Sequence Types				Housekeeping Gene Sequence Type						
Sequence Type	Total Number	Total rural	Total urban	*adk*	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*
58	6	5	1	6	4	4	16	24	8	14
10	4	4	0	10	11	4	8	8	8	2
155	4	3	1	6	4	14	16	24	8	14
101	2	2	0	43	41	15	18	11	7	6
118	2	0	2	31	4	42	44	15	33	17
357	2	0	2	13	40	13	13	23	25	66
642	2	1	1	9	23	33	18	11	8	6
720	2	0	2	35	3	58	6	5	16	4
2354	2	0	2	6	6	5	26	7	8	14
2521	2	1	1	6	19	3	135	11	8	6
2766	2	2	0	6	4	5	1	11	8	6
6032	2	0	2	6	23	1	1	9	13	6
13	1	0	1	6	6	5	9	9	8	2
23	1	1	0	6	4	12	1	20	13	7
43	1	0	1	24	11	4	8	8	8	2
69	1	0	1	21	35	27	6	5	5	4
111	1	1	0	6	29	14	16	24	8	2
127	1	0	1	13	14	19	36	23	11	10
154	1	1	0	6	6	5	10	9	8	6
162	1	0	1	9	65	5	1	9	13	6
196	1	1	0	6	19	3	16	9	8	6
224	1	0	1	6	4	33	16	11	8	6
224	1	0	1	6	4	33	16	11	8	6
296	1	0	1	9	23	64	77	11	8	6
336	1	1	0	9	4	33	18	11	8	6
345	1	1	0	6	4	14	1	20	62	7
398	1	1	0	64	7	1	1	8	8	6
423	1	1	0	6	4	33	1	20	12	7
543	1	0	1	83	136	110	117	80	1	2
635	1	0	1	6	107	1	95	69	8	7
645	1	1	0	117	148	120	12	80	1	2
711	1	1	0	9	6	15	131	24	7	7
1011	1	1	0	6	4	159	44	112	1	17
1081	1	0	1	6	4	5	18	11	8	2
1123	1	1	0	6	6	5	1	9	8	6
1125	1	1	0	6	4	15	18	24	26	7

1140	1	1	0	83	23	164	181	80	1	42
1304	1	1	0	6	95	4	18	11	7	14
1308	1	0	1	6	6	33	1	24	8	7
2165	1	0	1	6	23	3	16	9	7	7
2329	1	1	0	9	6	33	18	7	8	7
2541	1	0	1	148	29	33	16	11	8	41
2766	1	1	0	6	4	5	1	11	8	6
6552	1	0	1	13	44	49	13	16	10	15
6595	1	1	0	6	4	3	88	7	8	6
8926	1	1	0	126	160	131	6	5	679	99
10*	1	0	1	10	11	4	8*	8	8	2
607*	1	0	1	113	4	5*	83	8	8	6

*Novel Allele, ST may indicate nearest ST; **Alleles with less than 100% identity found

Multi-locus sequence typing (MLST) was performed using the Center for Genomic Epidemiology MLST 2.0 tool [29]. *E. coli* sequence typing #1 scheme was used to define the sequence type using seven conserved housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) to identify the sequence type and illustrate the genetic relationship [30].

INTRODUCTION

Antimicrobial resistance (AMR) is a significant public health concern that complicates the prevention and treatment of infectious diseases. This sustained emergence of AMR and an increase in selective pressures on bacteria for antibiotic resistance genes (ARGs) is associated with the use and misuse of antibiotics in healthcare and agricultural settings [1]. In healthcare settings, prolonged antibiotic use is a significant factor contributing to AMR, which undermines the control of nosocomial outbreaks caused by antimicrobial-resistant bacteria (ARB), such as methicillin-resistant *Staphylococcus* aureus [2,3]. Additionally, recent literature has identified an increase of AMR among community-acquired infections [4]. Extended-spectrum beta-lactamase (ESBL) producing bacteria are historically associated with hospital-acquired infections. However, recent increases in documented cases of community-acquired infections have raised questions about the impact that the environment has in the distribution of ARB and ARGs [5,6,7]. This observed transmission of AMR *E. coli* to humans from environmental sources can help explain community-associated AMR infections in patients without healthcare-associated risk factors [8].

Potential anthropogenic sources of ARGs in the environment include wastewater treatment plants and animal feeding operations that discharge waste into waterways, such as rivers and streams [9,10]. Also, antimicrobial-resistant *E. coli* and other Gram-negative bacteria are present at elevated levels in effluent downstream of hospital wastewater discharge compared to raw wastewater discharge [11]. This highlights the public health concern with discharging hospital wastewater into the municipal wastewater treatment system, which eventually leads to the environment [12]. Additionally, in agricultural settings, water systems

downstream of animal feeding operations have higher proportions of ESBL-producing *E. coli* [9,13,14]. Further research is needed to assess the difference in ARG prevalence in distinct watersheds, such as rural and urban, given that rivers and streams are an essential reservoir for ARB and ARGs in the environment [15,16]. This information would equip clinicians and public health officials with data to assess the epidemiologic risk and consequently support effective prevention and control of AMR infections, such as ESBL-producing *E. coli.*

E. coli is a sentinel bacterium to describe the prevalence of ARGs within an environment, because of its exceptional ability to acquire DNA through horizontal gene transfer and its overall ubiquity within the human, animal, and environmental microbiota [17,18]. For these reasons, infections of drug-resistant *E. coli* strains, such as ESBL-producing *E. coli*, pose unique challenges to the epidemiology in understanding the dissemination of ARGs and the public health risk of prevalent ARGs in the environment [19,20]. This retrospective descriptive study describes the presence of ARGs in *E. coli* isolated from water samples from two distinct watersheds, urban and rural, with the use of whole-genome sequencing (WGS). WGS data of the *E. coli* isolates is annotated for ARGs to identify genes responsible for phenotypic AMR expression and to assess the predictability for antimicrobial susceptibility testing using the detection of ARGs. Additionally, multi-locus sequence typing with WGS data for the *E. coli* isolates is used to describe the isolates and better understand the distribution of strains.

METHODS

Study Setting

The genomic and environmental samples were collected by Dr. Tanner and colleagues from the University of Utah, in order to assess the ARGs and factors associated with the presence of such ARGs in river systems [21]. *E. coli* isolates were cultured from water samples from two distinct watersheds, rural and urban. Watersheds were chosen for their substantial inputs from animal sources or human sources, respectively. The animal watershed, referred to as the rural watershed, was chosen based on the considerable concentration of animal feeding operations and the minimal concentration of human inputs, such as wastewater treatment plants WWTPs. In contrast, the human watershed, referred to as the urban watershed, was chosen based on the concentration of WWTP and the absence of animal feeding operations [21].

The Rock River was selected as the rural watershed, and the Blue River was selected as the urban watershed. *E. coli* isolates were obtained from environmental water samples from 46 sites within these two watersheds. Twenty-two rural sites from the Rock River yielded 73 rural watershed *E. coli* isolates collected in June 2017, and 24 urban sites from the Blue River yielded 70 urban watershed *E. coli* isolates collected in August 2017. The rural watershed, the Rock River, is characterized by being a primarily agricultural watershed located in southwest Minnesota and northwest Iowa. There are approximately 2,200 animal feeding operations within the catchment area of the watershed. There are 10 WWTPs, serving 19,119 people, and four hospitals located in the watershed. The urban watershed, the Blue River, runs between Kansas City, KS, and Independence, MO. There are six WWTPs, serving 534,901 people, and 20

hospitals in the catchment area of the watershed. There are no animal feeding operations in the watershed.

AMR susceptibility testing and WGS annotation for resistance genes

E. coli isolates were cultured from environmental water samples collected from the urban and rural watersheds. There was one sample per sample site, which was used to inoculate one plate. From each inoculated plate , up to three *E. coli* colonies were randomly selected for phenotypic testing. A total of 143 isolates were tested for phenotypic resistance using the Kirby-Bauer method to determine resistance profiles for gentamicin, ceftazidime, cefotaxime, aztreonam, ceftriaxone, cefazolin, cefoxitin, cefepime, ampicillin, amoxicillin with clavulanic acid, meropenem, ciprofloxacin, tetracycline, and sulfamethoxazole-trimethoprim [22]. Two antimicrobial susceptibility testing thresholds were used for cefazolin following the 2018 Clinical and Laboratory Standards Institute (CLSI) guidelines [22]. One used for systemic infections and one used for uncomplicated urinary tract infections. Broth A microdilution plates were used to determine the minimum inhibitory concentration (MIC) of colistin for each isolate. The results of the colistin resistance profiles for the samples were interpreted using the 2020 CLSI criteria [23]. Isolates that tested susceptible to an antibiotic by phenotypic antimicrobial susceptibility testing were considered susceptible to the antibiotic, and isolates that tested resistant or intermediate were considered non-susceptible to the antibiotic. Additionally, isolates were phenotypically tested for the production of ESBLs following the clavulanic acid test included in the 2018 CLSI criteria [22]. The categorical variable of rural and urban watersheds was assessed using Fisher's exact test, and the two-tailed p-value of <0.05 was considered statistically significant.

A subset of seventy-eight isolates from the water samples was selected for WGS because they expressed phenotypic AMR to one or more antibiotics. Sixty-eight samples were used in this report to observe the ARGs among *E. coli* from environmental water samples, eight samples were not available at the time of this report, and two WGS samples did not pass quality thresholds during *de novo* assembly. Samples were sequenced using the MiSeq® platform (Illumina, San Diego, CA) at the University of Utah and Huntsman Cancer Institute High Throughput Genomics Center, and reads were processed utilizing assembly and annotation tools from PATRIC: The Bacterial Bioinformatics Resource Center (University of Chicago) [24,25]. Annotation for ARGs was conducted using the PATRIC annotation tool, yielding identified ARGs by the CARD database [26]. ARGs were further filtered based on antimicrobial type using the Structured Antimicrobial Resistance Genes (SARG) v2.0 database [27]. Microsoft Excel (2016) and R studio with R version 3.5.1 (2018) were used for the statistical analysis and data management for this study [28].

Prediction of Phenotypic Antimicrobial Susceptibility Testing

Susceptibility predictions were assessed using the 68 isolates WGS and their antimicrobial susceptibility profiles. ARGs associated with phenotypic non-susceptibility were used to predict susceptibility profiles to their associated antibiotic or antibiotic classes. From these models, sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were calculated. Susceptibility prediction models were performed for ARGs identified by the SARG v2.0 database that were associated with antibiotics or a class of antibiotics that were used for antimicrobial susceptibility testing [27].

Multi-locus Sequence Typing

Multi-locus sequence typing (MLST) was determined using the Center for Genomic Epidemiology MLST 2.0 tool [29]. *E. coli* sequence typing #1 scheme was used to define the sequence type [30]. This MLST scheme uses seven conserved housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) to identify the sequence type and illustrate the genetic relationship.

RESULTS

Characteristics of Isolates

This observational study explored the phenotypic and genotypic antimicrobial resistance profiles of *E. coli* isolates obtained from environmental water samples collected from rural and urban watersheds. In total, 143 *E. coli* were obtained from environmental water samples from 46 sites within the two watersheds, 73 *E. coli* isolates from 22 rural sites from the Rock River, and 70 *E. coli* isolates from 24 urban sites from the Blue River.

Antimicrobial Resistance Patterns

Among the 143 *E. coli* isolates the prevalence of antimicrobial non-susceptibility from highest to lowest was cefazolin for systemic treatments (82.5%), ampicillin (23.1%), amoxicillin with clavulanic acid (14.0%), tetracycline (12.6%), cefoxitin (5.6%), trimethoprim-sulfamethoxazole (4.9%), ceftriaxone (2.8%), cefotaxime (2.1%), cefazolin for uncomplicated urinary tract infections (2.1%), cefepime (1.4%), ceftazidime (1.4%), aztreonam (1.4%), meropenem (1.4%), and ciprofloxacin (0.7%) (Table 1). All isolates were susceptible to gentamicin. Following 2020 CLSI criteria, colistin resistance was identified in four *E. coli* isolates, three from the rural watershed, and one from the urban watershed [23]. Among the 143 *E. coli* isolates in this analysis, we identified two isolates as ESBL-producing *E. coli* by the clavulanic acid test [22]. Within the 143 *E. coli* isolates, there was a significant difference in prevalence for tetracycline non-susceptibility comparing watersheds (Table 1). *E. coli* isolates from the rural watershed had a significantly higher prevalence of phenotypic non-susceptibility for tetracycline (21.9% vs. 2.9%, $p < 0.01$) when compared to the urban watershed isolates.

Resistance Genes

Among all 68 isolates sequenced, 37 from the rural watershed, and 31 from the urban watershed, the SARG v2.0 database identified 40 specific ARGs. All isolates contain one or more ARG classified as a gene conferring multidrug resistance. The distribution of ARG stratified by ARG type are bacitracin (98.5%), polymyxin (97.1%), tetracycline (19.1%), beta-lactam (11.8%), sulfonamide (10.3%), trimethoprim (4.4%), chloramphenicol (2.9%), fosmidomycin (1.5%), macrolide-lincosamide-streptogramin (1.6%), and unclassified (1.6%) (Table 2). Among the 68 isolates, there is no ARG recognized from the SARG v2.0 database ARG types of aminoglycosides, bleomycin, carbomycin, fosfomycin, fusaric-acid, fusidic-acid, kasugamycin, puromycin, quinolone, rifamycin, spectinomycin, tetracenomycin, and vancomycin. Given the selection process of WGS isolates, Fisher's exact test was not considered appropriate in evaluating ARG prevalence by watershed type.

Rural watershed isolates contained more variability of ARGs with 10 ARG classes represented compared to 7 ARG classes represented in urban watershed isolates. Within two rural watershed isolates, there was one ARG, *flor,* associated with chloramphenicol resistance. Within one rural watershed isolate, there were two ARGs, *macA* and *samples,* conferring macrolide-lincosamide-streptogramin resistance. Additionally, *rosA,* an ARG associated with a fosmidomycin resistance protein, was present in another rural watershed isolate. ARGs representing chloramphenicol, macrolide-lincosamide-streptogramin, and fosmidomycin resistance classes were not identified in urban watershed *E. coli* isolates. One ARG, *sdiA,* was only identified in one urban watershed *E. coli* isolate.

Antimicrobial Resistance Genes and Prediction of Phenotypic Antimicrobial Susceptibility Testing

The bacitracin resistance gene, *bacA,* was genotypically identified by the SARG v2.0 database in all isolates (n = 68). All isolates in the sample contained five or more ARG identified as conferring multidrug resistance, with 27 unique multidrug ARGs. The number of multidrug ARGs in each isolate ranged from 5 to 27, with an average of approximately 24 ARGs in each isolate. All isolates except for two contained an ARG, *arnA*, conferring polymyxin resistance.

Two beta-lactam resistance genes, *TEM-1* or *CMY-2*, were identified in 8 (11.8%) *E. coli* of the 68 isolates, five from the rural watershed, and three from the urban watershed. Seven of the eight isolates had the beta-lactam ARG *TEM-1* present, five of which were from the rural watershed, and two of which were from the urban watershed. *TEM* ARGs can be an ESBL-associated gene and thus indicates potential ESBL-producing *E. coli* within the environment [31]. *CMY-2* was present in a single urban watershed isolate.

Using the SARG v2.0 database's detection of genotypic resistance, the beta-lactam resistance genes, *TEM-1* and *CMY-2,* were used to predict isolates non-susceptible to the beta-lactam antimicrobial cephalosporins, such as cefazolin, cefoxitin, cefotaxime, ceftazidime, ceftriaxone, and cefepime, and penicillin antibiotics, ampicillin and amoxicillin with clavulanic acid. The accuracies of correctly classifying the non-susceptibility to cephalosporin antimicrobials in the 68 *E. coli* isolates using the ARGs, TEM-1 or CMY-2, were high, with a range of 79.4% - 100.0% when using the 2018 CLSI uncomplicated urinary tract infection susceptibility thresholds for cefazolin. Using the presence of the ARG *CMY-2* to predict non-susceptibility to ceftazidime was 100.0% accurate within the 68 *E. coli* isolates, but it is important to note, there

was only one *E. coli* isolate with the ARG, *CMY-2* (Table 3). The ARG *CMY-2* was 67.6% and 77.9% accurate when used to predict non-susceptibility to ampicillin and amoxicillin with clavulanic acid, respectively (Table 3).

For the ARG *TEM-1,* there was a 100.0% sensitivity for detecting ampicillin non-susceptibility and for detecting cefazolin non-susceptibility when using the systemic infection 2018 CLSI susceptibility thresholds for cefazolin [22]. The accuracy of using *TEM-1* to predict non-susceptibility for cefazolin when using the systemic infection susceptibility thresholds was 26.5%. Accuracy was highest for correctly classifying antimicrobial susceptibility testing for ceftazidime and cefepime at 88.1% each (Table 3). The accuracies for the prediction of non-susceptibility for ampicillin and amoxicillin with clavulanic acid were 76.5% and 77.9%, respectively (Table 3).

Tetracycline ARGs were identified in 13 (19.1%) *E. coli* of the 68 isolates, 12 isolates originating from the rural watershed and one isolate originating from the urban watershed, and consisted of *tetC* (n=9), *tetA* (n=4), *tetR* (n=4), and *tetD* (n=3) respective to abundance in the samples of isolates. Nine rural watershed isolates had one tetracycline ARG, three rural watershed isolates contained three tetracycline ARGs, and the one urban watershed isolate contained two tetracycline ARGs. Using tetracycline resistance genes to predict phenotypic non-susceptibility for tetracycline had a sensitivity of 100.0% for each gene (Table 3). The ARG *tetC* was the most accurate in predicting tetracycline susceptibility profiles of the isolates at 89.7%, and the ARG *tetD* was the least accurate of the tetracycline resistance genes identified in the 68 *E. coli* isolates at 79.4% (Table 3).

A sulfonamide ARG, *sul2* was observed in 7 isolates, six rural watershed isolates, and one urban watershed. Additionally, three rural watershed isolates with *sul2* also contained the ARG *dfrA5* associated with trimethoprim resistance. Using the ARGs of *sul2* and *dfrA5* independently to predict phenotypic non-susceptibility for trimethoprim-sulfamethoxazole, there was 100.0% and 85.7% sensitivity when using *dfrA5* and *sul2*, respectively (Table 3). The accuracies of correctly classifying susceptibility profiles for trimethoprim-sulfamethoxazole among the 68 *E. coli* isolates were 98.5% and 95.6% when using *sul2* and *dfrA5*, respectively (Table 3).

Multi-locus Sequence Typing

Multi-locus sequence typing analysis identified 47 unique sequence types following the *E. coli* #1 Scheme [30]. Dominant sequence types within the 68 isolates were ST58 (n=6, 8.8%), ST10 (n=5, 7.4%), and ST155 (n=4, 5.9%). Five rural watershed isolates and one urban watershed isolate represented ST58, four rural watershed isolates represented ST10, and three rural watershed isolates and one urban watershed isolate represented ST155. Additionally, there was an *E. coli* isolate from the urban watershed with six of seven alleles for ST10. Sequence types ST101, ST118, ST357, ST642, ST720, ST2345, ST2521, ST2766, and ST6032 (n=2, 3.1%) appeared in two isolates (Table 4). Isolates of the same sequence types varied in their phenotypic and genotypic antimicrobial susceptibility profiles.

DISCUSSION

Antimicrobial resistance is an emerging public health issue that is prevalent in healthcare and community settings. An increased reporting of AMR in community-acquired infections emphasizes the need for further research to understand the epidemiological implications of environmental AMR [4,8]. This retrospective observational study sought to describe AMR among environmental *E. coli* isolated from samples collected from two separate watersheds, representing rural and urban environments. Major findings and products of this report are the presence of important epidemiological ARGs in the environment, the non-susceptibility trends of rural and urban watershed *E. coli*, the application of WGS to predict non-susceptibility, and the use of whole-genome MLST to compare bacteria.

The presence of beta-lactam antimicrobial resistance in the *E. coli* isolates from environmental water samples is of public health concern, given the use of beta-lactam drugs to treat human-pathogens. Among the 143 *E. coli, t*here was a notable high level of non-susceptibility to cefazolin (85.9%) when using the susceptibility threshold for systemic infections, which is supported by current trends of rising cefazolin resistance [32]. Additionally, there was a higher prevalence of beta-lactam resistance, such as cefoxitin and ceftazidime, within the urban watershed isolates when compared to the rural watershed isolates (Table 1). One possible factor contributing to this higher prevalence of beta-lactam non-susceptibility observed in the urban watershed is the greater number of hospitals, 20, contained in the urban Blue River watershed catchment area, compared to four hospitals in the rural Rock River watershed catchment area. Hospital wastewater is associated with increased levels of antimicrobial-resistant *E. coli* and other gram-negative bacteria compared to raw wastewater

discharge [11,12]. This dissemination of antimicrobials and ARGs from hospital wastewater effluent into waterways can help explain the prevalence of beta-lactam resistant *E. coli* within the water samples.

The ARGs, *TEM-1* and *CMY-2,* which are associated with beta-lactam resistance, were identified within the *E. coli* isolates. *TEM-1* is a beta-lactamase-producing gene that is associated with resistance to penicillin and cephalosporins, supporting our observation that all isolates harboring *TEM-1* demonstrated phenotypic non-susceptibility to ampicillin and cefazolin (Table 3) [33]. Additionally, a mutation in *TEM-1* can alter the amino acid configuration around the enzyme active site allowing for ESBL production [31]. Secondary testing and genetic analyses are needed to determine if isolates with *TEM-1* variants produce ESBL. WGS and molecular detection methods are essential in further describing and supporting conventional susceptibility testing in detecting ESBL subtypes, highlighting the importance of accurate AMR testing to monitor and track potential epidemiologically important ARBs and ARGs, such as beta-lactam resistance genes [31]. Beta-lactam resistance genes, such as *TEM-1* and *CMY-2*, are a public health risk when identified in clinical or environmental settings, because of the observed and reported dissemination of resistance and the contribution to antibiotic treatment failure [7,11,13]. For these reasons, Martinez et al. have deemed beta-lactam resistance genes at the highest level of resistance readiness condition, RESCon 1, for their risk to public health relative to their environment [34].

The prevalence of phenotypic non-susceptibility and ARGs for tetracycline (12.6%) and trimethoprim-sulfamethoxazole (4.9%) within the 143 *E. coli* isolates are at lower than other prevalences reported in a review of AMR in environmental isolates of *E. coli* [20]. Additionally,

the higher prevalence of tetracycline non-susceptibility among the rural watershed isolates was statistically significant when compared to the urban watershed *E. coli* isolates (Table 1). WGS and ARG annotation identified *tetC, tetA, tetR, tetD*, *sul2,* and dfrA5 among the 68 sequenced isolates. These ARGS have also been observed in other river environments, notably the Yangtze River Delta in China [35]. This high prevalence of tetracycline non-susceptible *E. coli* and tetracycline resistance genes in the rural watershed can be explained by the higher concentration of animal feeding operations that contribute to wastewater effluent into the watershed [36]. Antibiotics such as tetracycline, sulfamethoxazole, and trimethoprim are used in animal feeding operations to promote growth and treat sick animals, which in turn increases the selective pressure for AMR in bacteria in these settings [37]. These same classes of antibiotics have been observed in urban environments, such as the effluent of urban residential areas, hospitals, and wastewater treatment plants, challenging the treatment of community-acquired urinary tract infections [20, 38].

Whole-genome sequencing and annotation for ARGS with the SARG v2.0 database were utilized to predict antimicrobial susceptibility testing results for the 68 sequenced isolates [27]. The overall accuracies of predicting non-susceptibility to antibiotics and antibiotic classes with ARGs were 82.6% for tetracycline non-susceptibility, 97.1% for trimethoprim-sulfamethoxazole non-susceptibility, 92.0% for non-susceptibility to cephalosporins, and 75.0% for non-susceptibility to penicillin antimicrobials. The overall accuracies for predicting antimicrobial non-susceptibility to cephalosporin drugs when stratifying by the genes, *TEM-1* and *CMY-2*, were 86.3% and 97.4%, respectively, which is most likely due to the presence of one identified *CMY-2* ARG among the isolates and the generally low levels of cephalosporin non-susceptibility.

These accuracies of predicting antimicrobial susceptibility for each class of antibiotics were consistent with reported accuracy percentages from other studies [39, 40,41]. Furthermore, approximately all prediction models had a sensitivity of 100.0%, which indicates the potential use of WGS to predict non-susceptibility profiles.

One limitation of this approach is that there is an incomplete understanding of all ARGs responsible for the observed phenotypes [40]. Furthermore, there could be multiple genes responsible for a single phenotypic expression, as well as a single ARG responsible for multiple non-susceptibility phenotypic expressions to multiple antimicrobial agents. The genotypic and phenotypic relatedness is complex. It comprises confounding factors that may not be measured or accounted for within the model, such as mutations of host genes that may synergistically or antagonistically affect phenotypic non-susceptibility expressions [39].

An additional limitation is the sample size and biased sampling of non-susceptible bacteria. A more varietal mixture of phenotypic expressions would more accurately represent the performance of WGS to predict antimicrobial susceptibility testing results. In the case of this study and the biased selection of *E. coli* non-susceptible to antimicrobials, the sensitivity and specificity values in predicting non-susceptibility with ARGs would be affected. For instance, this biased selection process would decrease the number of susceptible isolates within the subset. This, in turn, would decrease true negative and false negative values. By excluding isolates that are susceptible to antimicrobials, there would be a bias to increase sensitivity and decrease specificity. In interpreting the results of the performance of genotypic resistance data for the prediction of phenotypic antimicrobial non-susceptibility, it is crucial to

consider that sensitivity results may be greater and the specificity results may be less than the sensitivity and specificity of an unbiased sample of the *E. coli* isolates (Table 3).

WGS can also be used to identify sequence types of bacteria. Multi-locus sequencing typing is an essential tool for the surveillance of bacteria and the investigation of outbreaks, allowing for the description of the genomic relationship of sequence types. The dominant sequence types represented in the 68 isolates are ST58 (n=6), ST10 (n=5), and ST155 (n=4) (Table 4). Three rural watershed isolates of the six ST58 isolates had the same ARG and susceptibility profiles as ST58 *E. coli* isolates collected from the urine and blood of a patient observed in another study reporting on urosepsis [42]. These ST58 *E. coli isolates* have *drfA5, sul2,* and *TEM-1* ARGs, which could potentially confer non-susceptibility to trimethoprim, sulfamethoxazole, cephalosporins, and penicillin antimicrobial agents. This resistance profile is associated with commensal and pathogenic *E. coli* isolated from animals and humans [42, 43, 44]. The presence of pathogenic and AMR *E. coli* in environment reservoirs such as waterways may indicate the sustained selection pressure for such ARGs and the promotion of clinically significant pathogenic *E. coli* in the environment [14].

Other sequence types such as ST10 and ST155 have been described in other reports looking at colistin resistance in *E. coli* in agricultural environments in Algeria [45]. ST10 and ST155 isolates in this descriptive study were not resistant to colistin, and they do not have colistin resistance genes, *mcr-1,* and *mcr-3,* which were observed in similar descriptive studies on these sequence types [45]. This discrepancy is dependent on the setting, selective pressure, and prevalence of colistin resistance in the local environment, such as the possible use of colistin in farming practices in Algeria. This difference highlights the indiscriminate nature of

sequence types and the need to understand confounding variables within the environment and the microbial community. One isolate of epidemiological interest shared a sequence type, ST224, with a highly virulent ESBL-producing *E. coli* reported causing pneumonia in a domestic cat [46]. This urban isolate of ST224 contains the ARG, *CMY-2,* and has a similar phenotypic non-susceptibility profile like the pneumonia-causing ST224 *E. coli,* which was non-susceptible to all beta-lactams tested except for cefepime [46]. These phenotypic susceptibility testing results are identical to sequence types present in various animal-human interface environments, illustrating the complex, multifaceted dissemination pathways within the environment of ARGs [46,47,48].

A significant limitation in the interpretation and the assessment of the potential public health risk of the AMR and specific ARGs identified in the 68 *E. coli* isolates is that the pathway from water environments to human environments is complex and involves multiple bottlenecks modulating bacteria and gene transfer [34,49,50]. These bottlenecks related to ecological connectivity, fitness costs, and further selection will ultimately determine the establishment of ARGs in a population of pathogenic bacteria [49]. Consequently, these ARGs and beta-lactam non-susceptible *E. coli* isolates can be viewed as epidemiologically crucial for tracking and describing AMR in the environment. Another limitation of this retrospective observational study is that the selection process of isolates for whole-genome sequencing was not randomized; instead, it was dependent on phenotypic antimicrobial susceptibility testing. This limits the utilization of statistical analyses of ARGs and the *E. coli* isolates, as well as the interpretation of sensitivity and specificity values in predicting antimicrobial non-susceptibility with WGS. Additionally, there are potential confounder variables, such as metal and biocide

concentrations, which can be assessed for possible associations to antimicrobial susceptibility profiles of the *E. coli* isolates. Both metal concentrations and biocides have been associated with AMR in the environment [51,52]. For these reasons, this study is not able to draw strong associations of observed results and related variables, such as study site. Furthermore, the generalizability of the findings of this observational study is limited to similar watersheds and phenotypic profiles of *E. coli* isolates. These limitations highlight the necessity for further research on AMR within watersheds utilizing WGS.

Further research on the systems and pathways involved in the dissemination of ARGs between environments is necessary to estimate the health risk associated with ARGs and ARBs present in aquatic environments. Additionally, further data processing, such as annotation of plasmids from the WGS, will help describe possible sources and relationships of AMR within the watershed environment [53]. More so, investigating the co-occurrence of ARGs detected in the isolates to better describe the specific nuances of AMR in the environment would yield a better understanding of ARB and ARGs in water environments.

CONCLUSION

In conclusion, this study describes the antimicrobial resistance and epidemiology of *E. coli* isolated from the rural Blue river and urban Rock River watersheds. This report has demonstrated the prevalence of diverse and abundant ARGs and ARB within both watersheds, including epidemiologically significant ARGs and *E. coli* sequence types. Waterways are vital reservoirs and disseminators of ARB and ARGs, as demonstrated by the reported observations in this paper. The resistance profiles of *E. coli* within this study can pose a risk to public health and infectious disease treatment. Further research and monitoring of AMR and ARGs in aquatic environments are necessary to improve health through the understanding of the dissemination of AMR in the environment. With more knowledge of AMR in the local environment, public health officials and clinicians can more effectively prevent, control, and treat AMR infections.

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