



Extended Spectrum β -Lactamase Activity and Cephalosporin Resistance in *Escherichia coli* from U.S. Mid-Atlantic Surface and Reclaimed Water

Sultana Solaiman,^a Eric Handy,^b Taylor Brinks,^a Kasey Goon,^a Chris Bollinger,^a Amy R. Sapkota,^c  Manan Sharma,^b  Shirley A. Micallef^{a,d}

^aDepartment of Plant Science and Landscape Architecture, University of Maryland, College Park, Maryland, USA

^bEnvironmental Microbial and Food Safety Lab, USDA-ARS, Beltsville, Maryland, USA

^cMaryland Institute of Applied and Environmental Health, School of Public Health, University of Maryland, College Park, Maryland, USA

^dCenter for Food Safety and Security System, University of Maryland, College Park, Maryland, USA

ABSTRACT Phylogenetic distribution and extended spectrum β -lactamase (ESBL) activity of *Escherichia coli* recovered from surface and reclaimed water in the mid-Atlantic U.S. were evaluated. Among 488 isolates, phylogroups B1 and A were the most and least prevalent, respectively. Water type, but not season, affected phylogroup distribution. The likelihood of detecting group A isolates was higher in reclaimed than pond ($P < 0.01$), freshwater river ($P < 0.01$) or brackish river ($P < 0.05$) water. Homogeneity in group distribution was lowest in pond water, where group B1 comprised 50% of isolates. Only 16 (3.3%) isolates exhibited phenotypic resistance to one or more cephalosporins tested and only four had ESBL activity, representing groups B1, B2 isolates, and D. Phylogroup was a factor in antimicrobial resistance ($P < 0.05$), with group A (8.7%) and D (1.6%) exhibiting the highest and lowest rates. Resistance to cefoxitin was the most prevalent. Multi- versus single drug resistance was affected by phylogroup ($P < 0.05$) and more likely in groups D and B1 than A which carried resistance to cefoxitin only. The most detected β -lactam resistance genes were *bla*_{CMY-2} and *bla*_{TEM}. Water type was a factor for *bla*_{CTX-M} gene detection ($P < 0.05$). Phenotypic resistance to cefotaxime, ceftriaxone, cefuroxime and ceftazidime, and genetic determinants for ESBL-mediated resistance were found predominantly in B2 and D isolates from rivers and reclaimed water. Overall, ESBL activity and cephalosporin resistance in reclaimed and surface water isolates were low. Integrating data on ESBL activity and β -lactam resistance among *E. coli* populations can inform decisions on safety of irrigation water sources and One Health.

IMPORTANCE Extended spectrum β -lactamase (ESBL) producing bacteria, that are resistant to a broad range of antimicrobial agents, are spreading in the environment but data remain scarce. ESBL-producing *Escherichia coli* infections in the community are on the rise. This work was conducted to assess presence of ESBL-producing *E. coli* in water that could be used for irrigation of fresh produce. The study provides the most extensive evaluation of ESBL-producing *E. coli* in surface and reclaimed water in the mid-Atlantic United States. The prevalence of ESBL producers was low and phenotypic resistance to cephalosporins (types of β -lactam antibiotics) was affected by season but not water type. Data on antimicrobial resistance among *E. coli* populations in water can inform decisions on safety of irrigation water sources and One Health.

KEYWORDS irrigation water, ESBL-producing *Escherichia coli*, β -lactam resistance, cephalosporin resistance, antimicrobial resistance genes, reclaimed wastewater for irrigation

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Address correspondence to Shirley A. Micallef, smical@umd.edu.

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Agricultural water is a major reservoir of known and non-reportable foodborne pathogens (1), driving raw vegetable associated foodborne illnesses that are increasingly traced back to irrigation water (2). Prospective and on-farm agricultural water sources in the mid-Atlantic United States, including river, pond and treated municipal wastewater (reclaimed water), harbor high concentrations of fecal coliforms and enteric pathogens (3–5). Assurance of microbiological quality of water for irrigation purposes is a critical step in minimizing crop contamination and support food safety efforts. These efforts should comprise in depth investigations through ecological studies that go beyond quantification of fecal bacteria. Assessment of other hazards, such as distribution of antimicrobial resistance gene carriage in pathogenic and non-pathogenic bacterial populations would further advance our comprehension of risk associated with specific water sources.

The World Health Organization (WHO) and the U.S. Food and Drug Administration (FDA) recommend *Escherichia coli* over other heterotrophic bacteria as a microbial water quality indicator for fecal contamination because of its natural residence in mammalian gastrointestinal tracts (6, 7). Current FDA standards for agricultural water in the Food Safety Modernization Act (FSMA) Produce Safety Rule (PSR; 21 CFR 112) use generic *E. coli* (8) when testing is needed. Although most strains of *E. coli* are commensal in the human gut, some are pathogenic and able to cause a variety of illnesses including gastrointestinal and extraintestinal diseases. Six well-characterized *E. coli* pathogenic groups known for causing gastroenteritis are enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC) and diffusively adherent *E. coli* (DAEC) (9).

Due to the extensive diversity in genetic substructure, *E. coli* is classified into four different phylogroups, A, B1, B2 and D, based on the presence of three virulence genes, heme transport (*chuA*), stress related gene (*yjaA*) and lipase/esterase (*tspE4C2*) (10). Phylogroup members share distinct phenotypic and genotypic characteristics. Generic/commensal strains make up most of phylogroups A and B1, in addition to some Shiga-toxin producing and entero-pathogenic strains in these two groups, respectively. Groups B2 and D mostly comprise of infectious extra-intestinal strains with the latter group also including some entero-pathogenic strains (10–14).

Antimicrobial resistance (AMR) to several broad and extended spectrum antimicrobials in commensal and pathogenic *E. coli* is a major contributor of infection and enhanced morbidity and mortality (15). Every year, approximately 2.8 million people are sickened by AMR infections, with 197,400 estimated cases in hospitalized patients and 9,100 estimated deaths in 2017 attributed to extended spectrum β -lactamase (ESBL) producing Enterobacteriaceae (16). Extended spectrum β -lactamases are enzymes produced by bacteria that degrade β -lactam antibiotics including penicillins and cephalosporins, and ESBL-producing bacteria can be resistant to multiple antimicrobials. About 17% of all ESBL-producing *E. coli* were predicted to be virulent based on the presence of genetic markers for virulence determinants in a study evaluating 170 ESBL-producing isolates from Dutch wastewater (17). It is estimated that more global deaths are associated with or attributable to antimicrobial-resistant *E. coli* than any other antimicrobial resistant pathogen (18). ESBL-producing *E. coli* in the environment can emanate from untreated municipal sewage effluent, untreated hospital effluent or treated effluent (19) and further spread to fresh produce via irrigation water (20). Therefore, regardless of the phylogenetic class of *E. coli*, AMR of agro-environmental strains is a public health concern. In view of the increased reports of ESBL-producing *E. coli* in vegetables (21–24), surveillance of extended spectrum AMR should be expanded to fresh crop production areas to better understand temporal and spatial dynamics of this public health concern.

In a previous study we conducted a longitudinal assessment of the microbial water quality of 11 irrigation water sources as possible alternatives to groundwater (5). The study sites were located within the Chesapeake Bay watershed in the mid-Atlantic United States, a region with a very dense urban population and highly mixed land use, including agricultural and conservation areas. The study sites comprised of surface (irrigation ponds and rivers) and reclaimed water which were sampled over 330 times

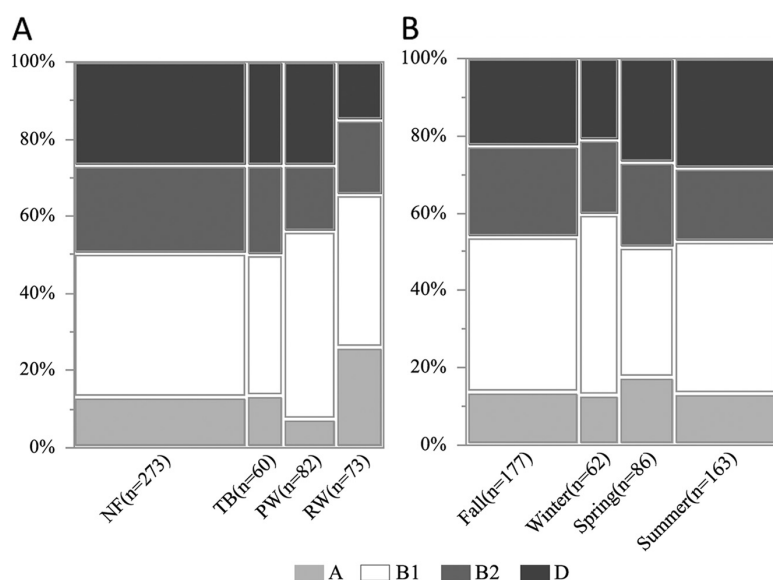


FIG 1 Distribution of different phylogenetic groups of *E. coli* by (A) surface and reclaimed water sources and (B) season. The mosaic plots display the % frequency in four water types: five non-tidal freshwater rivers (NF), one tidal brackish river water (TB), two on-farm ponds (PW), and three reclaimed water (RW) sites.

during a 2-year period and from which 724 *E. coli* isolates were isolated and archived for further characterization. The risk of introducing pathogenic and ESBL-producing *E. coli* to fresh produce crops via agricultural water could be water type dependent as a function of the heterogeneous distribution of *E. coli* phylogroups and ESBL-producing strains in water environments. We hence assessed *E. coli* phylogrouping among isolates and subsequently investigated ESBL activity and phenotypic and genotypic β -lactam mediated resistance, to extensively evaluate environmental *E. coli* in the mid-Atlantic region of the United States.

RESULTS

Phylogenetic classification of *uidA* $\pm$ *E. coli*. To avoid assessment of clonal isolates, isolates with a unique phylogroup and resistance profile per collection date and site were selected, yielding 488 isolates. A higher number of confirmed unique *E. coli* isolates were recovered in fall ($n = 177$) and summer ($n = 163$) than spring ($n = 86$) and winter ($n = 62$). Their distribution by water type was as follows: 273 from non-tidal freshwater rivers, 73 from tidal brackish rivers, 82 from pond and 60 from reclaimed water. Isolates were classified into 4 phylogroups: 193 isolates were classified as group B1 (39.6%), 122 isolates as group D (25.0%), 104 isolates as group B2 (21.3%) and 69 isolates as group A (14.1%). Site was a factor in phylogroup distribution (Pearson's χ^2 ($n = 488$, $df = 30$) = 53.82, $P < 0.01$). The freshwater river site MA04 had the highest percentage (17.4%) of group A isolates and the pond water site MA10 had the lowest (1.5%). MA09, also a freshwater river, harbored the highest shares (13%) of groups B1 and D (14%), while the lowest representation for both groups was from reclaimed water site MA06 (2%). Rivers MA05 and MA07 each contained about 14.4% of all group B2 isolates, while reclaimed water site MA01 had the lowest proportion (1.9%). The most uniform distribution of phylogroups within each site was observed at MA04, followed by MA08. Reclaimed water sites displayed substantial heterogeneity in phylogroup distribution compared to all other water types.

Water type was found to be a factor for phylogroup distribution (Pearson's χ^2 ($n = 488$, $df = 9$) = 17.15, $P < 0.05$; Fig. 1A). The likelihood of detecting group A isolates was higher in reclaimed water compared with pond water ($P < 0.01$), freshwater river ($P < 0.01$) or brackish river ($P < 0.05$) (Fig. 1A). The likelihood of detecting

TABLE 1 Antimicrobial susceptibility of 488 *E. coli* isolates recovered from surface and reclaimed water to select cephalosporin antibiotics^a

Antibiotic	% Resistant	% Intermediate	% Sensitive
Ceftazidime (CAZ)	1.0 (5)	0 (0)	99.0 (483)
Ceftriaxone (CRO)	1.4 (7)	0 (0)	98.6 (481)
Cefotaxime (CTX)	1.4 (7)	0 (0)	98.6 (481)
Cefuroxime (CXM)	1.6 (8)	0.2 (1)	98.2 (479)
Cefoxitin (FOX)	2.9 (14)	0 (0)	97.1 (474)

^aThe number of isolates (*n*) is given in parentheses.

members of group B1 was slightly higher in reclaimed ($P < 0.1$) compared with fresh-water river. In general, the most prevalent phylogroup retrieved from all water types was B1 and the least prevalent was A (Fig. 1). The equitability index was used as a measure of the evenness of distribution of the four phylogroups by water type. The index approached 1 for rivers and reclaimed water (~ 0.96), indicating homogenous distribution of phylogroups, but was lower for pond water (0.86). In pond water, group B1 made up 50% of the assemblage, compared to $<40\%$ for all the other water types. Seasonal variation exerted no effect on the distribution of phylogroups (Pearson's Chi-squared test: χ^2 ($n = 488$, $df = 9$) = 6.21, $P > 0.1$; Fig. 1B).

Phenotypic resistance against cephalosporins. We selected five 2nd and 3rd generation cephalosporins for susceptibility testing. Most *E. coli* isolates exhibited susceptibility to all cephalosporins tested (Table 1). Only 16/488 (3.3%) isolates exhibited resistance to single or multiple antibiotics. Only 4 isolates exhibited ESBL activity. Assigned phylogroup was a factor in whether resistance to any antibiotic was observed (Pearson's χ^2 ($n = 488$, $df = 3$) = 8.41, $P < 0.05$). Six of the 16 isolates were group A (6/69, 8.7% of group A), four each were in groups B1 (4/193, 2.1% of group B1) and B2 (4/104, 3.8% of group B2), and two were in group D (2/122, 1.6% of group D) (Table 2). Among the resistant isolates, phylogroup was a factor in MDR versus SDR (Pearson's χ^2 ($n = 16$, $df = 3$) = 10.0, $P < 0.05$) with MDR associated with groups D, B1 and B2 and SDR with group A. Resistance patterns are listed in Table 2. Of note, one B1, one B2 and both D isolates exhibited resistance to all five cephalosporins tested (Fig. 2, Table 2). The six resistant isolates from group A exhibited resistance only to cefoxitin, the antibiotic for which resistance was most prevalent.

TABLE 2 Phenotypes and genotypes of ESBL-producing *E. coli* isolates recovered from four different water types, non-tidal fresh river/creek water (NF), tidal brackish river water (TB), pond water (PW) and reclaimed water (RW)^a

Resistant phenotype	Phylogroup	Water type	Season of collection	Genotype
CAZ/CTX/CRO/CXM/FOX	B1	NF	Spring	<i>bla</i> _{TEM} <i>bla</i> _{CMY-1} <i>bla</i> _{CMY-2}
	B2	NF	Winter	<i>bla</i> _{CMY-2}
	D	NF	Winter	<i>bla</i> _{TEM} <i>bla</i> _{CMY-2}
	D*	TB	Winter	<i>bla</i> _{CTX-M} <i>bla</i> _{OXA-2}
CTX/CRO/CXM/FOX	B1*	NF	Fall	<i>bla</i> _{CMY-2}
CAZ/CTX/CRO/CXM	B2*	RW	Fall	<i>bla</i> _{CTX-M}
CTX/CRO/CXM	B2*	RW	Fall	<i>bla</i> _{CTX-M} <i>bla</i> _{TEM} <i>bla</i> _{SHV} <i>bla</i> _{CMY-1} <i>bla</i> _{OXA-1}
CTX/FOX	B1	NF	Summer	None
FOX	A	NF	Fall	<i>bla</i> _{CMY-2}
	A	NF	Fall	<i>bla</i> _{TEM} <i>bla</i> _{CMY-2}
	A	RW	Fall	<i>bla</i> _{CMY-2}
	A	RW	Summer	<i>bla</i> _{TEM} <i>bla</i> _{CMY-2}
	A	NF	Summer	<i>bla</i> _{TEM} <i>bla</i> _{CMY-2}
	A	NF	Summer	<i>bla</i> _{CMY-2}
	B1	PW	Fall	<i>bla</i> _{CMY-1}
	B2	NF	Summer	<i>bla</i> _{TEM} <i>bla</i> _{CMY-2}

^aAntibiotics tested were ceftazidime (CAZ), ceftriaxone (CRO), cefotaxime (CTX), cefuroxime (CXM) and cefoxitin (FOX). *, denotes confirmed ESBL activity.

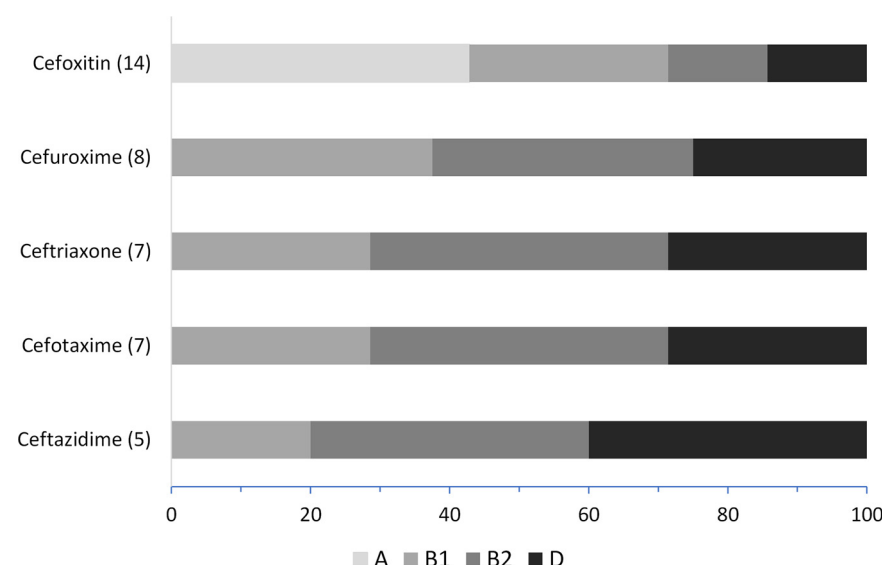


FIG 2 Cephalosporin resistance (% frequency) among 16 *E. coli* isolates by phylogroup. For each antibiotic, *n* denotes number of isolates resistant to that antibiotic.

Phylogroup was a factor for resistance to cefuroxime (Pearson's χ^2 ($n = 16$, $df = 3$) = 15.29, $P < 0.05$), ceftriaxone (Pearson's χ^2 ($n = 16$, $df = 3$) = 8.89, $P < 0.05$), cefotaxime (Pearson's χ^2 ($n = 16$, $df = 3$) = 8.89, $P < 0.05$) and ceftazidime (Pearson's χ^2 ($n = 16$, $df = 3$) = 7.86, $P < 0.05$). For all these antibiotics, resistance was more related to groups D and B2 than A.

Out of the 16 resistant isolates, 10 (62.5%) were retrieved from non-tidal freshwater river, 1 (6.3%) from tidal brackish river water, 1 (6.3%) from on-farm ponds and 4 (25.0%) from reclaimed water (Table 2). Of these, 1 of the freshwater river isolates, the tidal river water isolate and 2 of the reclaimed water isolates were ESBL-producing (Table 2). Although no significant effect of water type was detected on AMR among the 488 isolates, for individual antibiotics, ceftazidime resistance was somewhat more likely to be detected in isolates recovered from sites MA02 (reclaimed), MA08 (tidal river) and MA09 (freshwater river) (Pearson's χ^2 ($n = 488$, $df = 10$) = 16.45, $P < 0.1$). Resistant isolates were collected mostly in fall (43.8%, $n = 7/16$) and summer (31.3%, $n = 5/16$) but these differences were not statistically supported. Resistance to individual antibiotics appeared to be significantly impacted by season. Ceftazidime χ^2 ($n = 488$, $df = 3$) = 10.97, $P < 0.05$, cefotaxime χ^2 ($n = 488$, $df = 3$) = 7.58, $P = 0.05$ and ceftriaxone (Pearson's χ^2 ($n = 488$, $df = 3$) = 7.58, $P = 0.05$) resistance was more likely to be detected in winter. Seasonal distribution of resistance is given in Table 2.

β -lactam resistance genotypes. All 16 phenotypically resistant isolates were screened for seven β -lactam resistance genes: *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, *bla*_{CMY-1}, *bla*_{CMY-2}, *bla*_{OXA-1} and *bla*_{OXA-2}. A total of nine isolates (56.3%), representing all phylogroups, harbored two or more AMR genes and one group B1 isolate from freshwater river had none of the seven resistance genes (Table 2). Five resistance genes were accumulated in one B2 isolate collected from reclaimed water in fall. The most prevalent gene detected was *bla*_{CMY-2} (11/16, 68.8%) followed by *bla*_{TEM} (7/16, 43.8%), with both genes detected in *E. coli* isolates from all phylogroups.

Among the resistant isolates, water type was found to be a significant factor for detection of *bla*_{CTX-M} (Pearson's χ^2 ($n = 16$, $df = 3$) = 9.44, $P < 0.05$) and a weak association was seen for *bla*_{CMY-2} (Pearson's χ^2 ($n = 16$, $df = 3$) = 7.16, $P < 0.1$). Season was not determined to be an important factor except for a weak association with *bla*_{CMY-1} (Pearson's χ^2 ($n = 16$, $df = 3$) = 6.62, $P < 0.1$).

Association among phylogroups, their phenotypic and genotypic traits, and environmental variables. MCA was conducted on the 16 resistant isolates to assess the association between different variables: cephalosporin susceptibility pattern and AMR gene pattern in relation to phylogroup, season of isolate collection and water

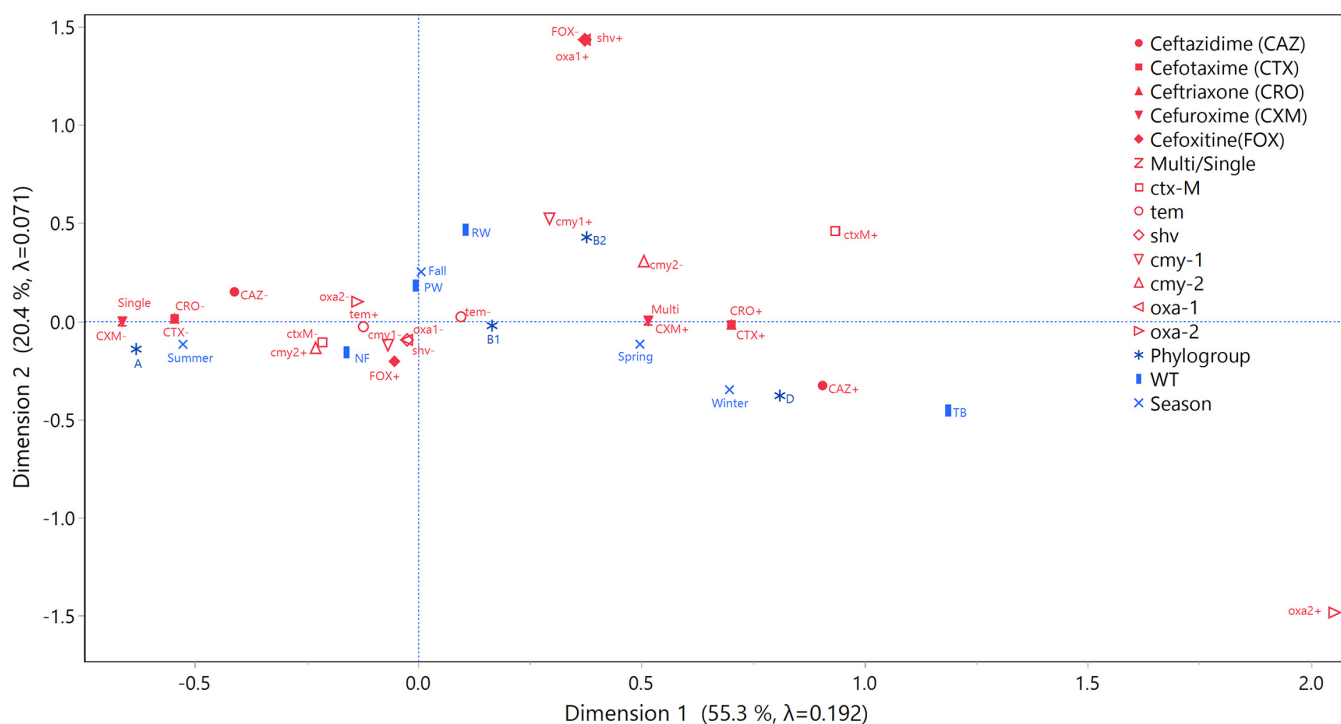


FIG 3 Multiple correspondence analysis (MCA) plot showing the relationships between phylogenetic groups of *E. coli* (number of isolates = 16), their phenotypic resistance traits and carriage of β -lactam antibiotic resistance genes, and season of collection and water source. Presence or absence (+/–) of each gene, *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, *bla*_{CMY-1}, *bla*_{CMY-2}, *bla*_{OXA-1}, *bla*_{OXA-2} were denoted as tem, shv, ctxM, cmY1, cmY2, oxa1 and oxa2, respectively.

type (Fig. 3). The two-dimensional MCA plot explained 75.7% of the variability. Water type and phylogroup contributed the most to the variability. Phylogroup A contributed 26% to inertia of dimension 1 while B2 contributed 22% to dimension 2. Brackish river water contributed 15% to inertia of dimension 1 and reclaimed water 26% to dimension 2 inertia. Winter and summer both contributed $\sim 15\%$ to dimension 1. In the MCA plot, objects plotting away from the origin of the plot are the most distinct, compared to objects plotting around the center. Cefotaxime, ceftriaxone and cefuroxime resistance plotted away from the center with MDR and were not related to summer collection when irrigation is at its peak use. Group A and summer were associated with SDR. Winter was related to isolates in group D and resistance to ceftazidime. Spring, summer and fall, pond and river water, and groups A and B1 were related to susceptibility to several antibiotics (except for cefoxitin) and absence of genes tested. The variables falling farthest from the center of the plot, representing traits of a minority of isolates, comprised the rarely detected genes *bla*_{SHV}, *bla*_{OXA-1} and *bla*_{OXA-2} and infrequently observed susceptibility to cefoxitin (Fig. 3).

Hierarchical cluster analysis of resistant *E. coli* isolates based on phenotypic resistance and resistance gene presence revealed two main branches, with isolates from groups A predominantly populating one cluster and groups B1, B2 and D comprising the other cluster (Fig. 4). The former cluster comprised only summer and fall isolates mostly from non-tidal freshwater river and reclaimed water sites. Isolates from group A showed the highest similarity among each other, independent of site or season of collection. The second cluster of B1, B2 and D groups was populated by isolates exhibiting MDR (Table 2). A pair of MDR ESBL-producers from reclaimed water and a tidal river that also carried the *bla*_{CTX-M} gene formed their own cluster (Table 2). ANOSIM supported differences by phylogroup (global $R = 0.48$, $P \leq 0.001$); specifically, between group A and B1 ($R = 0.71$; $P < 0.01$), A and B2 ($R = 0.49$; $P < 0.05$), and A and D ($R = 0.98$; $P < 0.05$). Water type was a determinant for β -lactam resistance (global $R = 0.30$, $P < 0.05$) but pairwise differences could not be determined due to small sample sizes for some water types. The seasonal effect was not significant ($R = 0.2$,

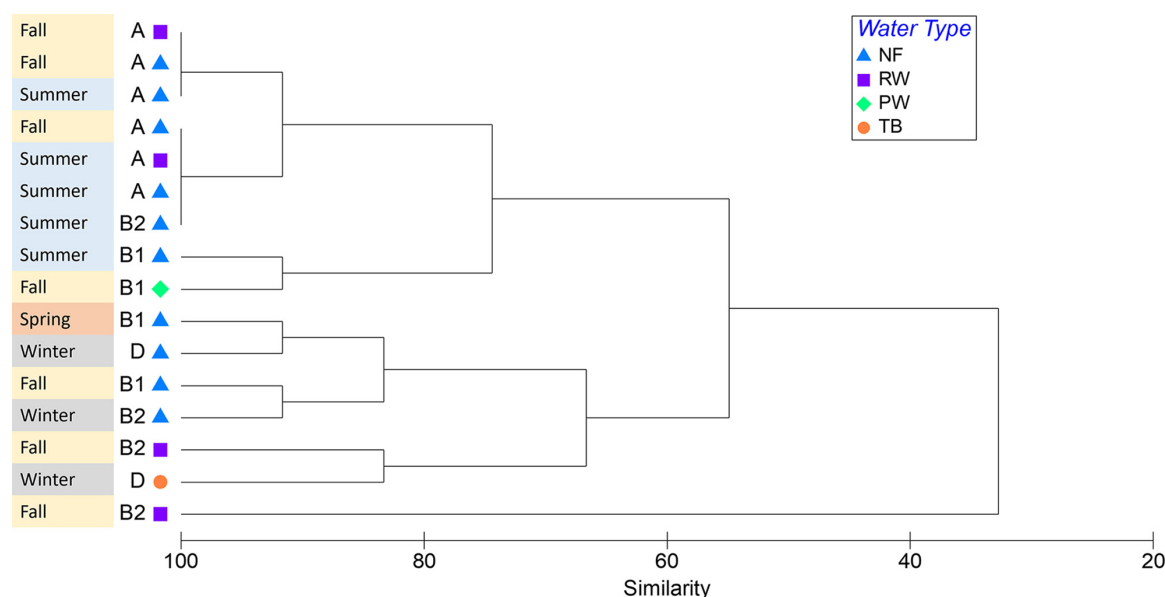


FIG 4 Hierarchical cluster analysis (HCA; group average method using simple matching coefficient) of resistant *E. coli* isolates based on profiles of phenotypic resistance to ceftazidime, ceftriaxone, cefotaxime, cefuroxime and cefoxitin, and genotypic resistance based on detection of genes *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, *bla*_{CMY-1}, *bla*_{CMY-2}, *bla*_{OXA-1} and *bla*_{OXA-2} by phylogenetic group (A, B1, B2 and D), water type (non-tidal freshwater rivers [NF], tidal brackish river [TB]; on-farm ponds [PW] and reclaimed water [RW]), and season of collection.

$P = 0.07$). Resistant isolates mostly derived from non-tidal freshwater river water and represented the most diversity, year round.

DISCUSSION

Demands for alternative and sustainable irrigation water for fresh fruit and vegetable production in the United States are redirecting attention from groundwater to reclaimed (recycled) and rechargeable surface water sources such as ponds and rivers. With the implementation of revised agricultural water standards to improve food safety, prioritizing irrigation water quality can minimize the risk of transfer of pathogenic and antimicrobial resistant *E. coli* to humans via fresh produce crops. Previous research by our group determined the adequacy of irrigation water sources in the mid-Atlantic based on *E. coli* concentrations and proposed microbial standards for agricultural water (5, 29). Research presented in this study was conducted to more specifically investigate the incidence of extended spectrum β -lactamase production and gene carriage, and cephalosporin resistance among *E. coli* in these irrigation water sources. Our study detected a year-round diversity of *E. coli* phylogroups in surface and reclaimed water sources, with phylogroup distribution exhibiting some dependency on water source. Despite this ubiquity and diversity, resistance to cephalosporins was low and only four isolates were confirmed ESBL-producers. While phylogrouping and season were factors affecting phenotypic resistance, the drivers influencing presence of β -lactamase genes were less clear. Conversely, an association between water type and phenotypic antimicrobial resistance was not detected but water type was a factor in *bla*_{CTX-M} gene detection. Integrating all the data revealed relationships among water type, season of collection, phylogroup, phenotypic resistance and β -lactamase gene carriage. Two main clusters of resistant *E. coli* isolates were identified, one comprised mainly of group A isolates with single drug resistance to cefoxitin and another cluster composed of several group B2 and D isolates carrying resistance to multiple cephalosporins.

E. coli isolates from irrigation water and farms in Mexico and Portugal mostly belonged to groups A and B1 (30, 31). Irrigation water, animals and humans in the mid-Atlantic United States previously yielded a higher proportion of group B1 compared to other genotypes of *E. coli* (32). Moreover, the lotic systems investigated in this study

were previously reported to carry higher microbial loads and higher levels of the food-borne pathogens *Salmonella* and *Listeria monocytogenes* compared to pond and reclaimed water (5). Hence, in this study, we anticipated group A and B1 dominance in farm ponds and some evidence for a differential food safety risk for rivers. However, *E. coli* phylogrouping revealed a homogenous distribution of groups in all water types, regardless of season. This study revealed that group B1 was the predominant group in all water types, but groups A and B1 were more likely to be detected in reclaimed and pond water, respectively. The lower equitability index in ponds was attributed to the predominance of group B1 in pond water, with a concomitant lower proportion of group A. Group D comprised a little over a quarter of all *E. coli* isolates in rivers and ponds, but only 15% of reclaimed water samples. Finally, rivers had a higher proportion of B2 isolates compared to pond and reclaimed water. In a pediatric study, phylogroup assignment of *E. coli* strains isolated from children found that group A isolates associated with asymptomatic cases, while group D associated with diarrhea (33). Isolates from groups B2 and D tended to cause persistent symptoms compared to milder diarrhea caused by groups A and B1 (33). Our findings, therefore, may signal a slightly higher likelihood of exposure to more severe intestinal pathogens (in groups B2 and D) from river water (average 50%), relative to ponds (44%) and reclaimed water (34%). Coupled with lower reported *S. enterica* and *L. monocytogenes* prevalence (5), the lower proportions of group B2 *E. coli* isolates in ponds, and B2 and D isolates in reclaimed water, support the preferential use of these water sources over rivers for irrigation of fresh crops.

The relatively low level of antimicrobial resistance to cephalosporins in *E. coli* detected in our various surface water sources and reclaimed water sites is encouraging for mid-Atlantic agriculture. The possibility of introducing antimicrobial resistant *E. coli* into the food chain via irrigated fresh produce crops is increasingly regarded as a plausible public health threat (34). Several studies have reported the occurrence of ESBL producers among the Enterobacteriaceae, including *E. coli*, isolated from vegetables (21, 23, 35–37). The presence of ESBL-producing bacteria in vegetables is especially worrying considering the occurrence of these bacteria in healthy humans, which appears to be spreading worldwide (38), and the potential for long-term persistence of these AMR bacteria in the agro-environment, once introduced (39). In addition to the low occurrence of ESBL-producing *E. coli* reported here, the phylogroup most likely to exhibit phenotypic resistance in our study was group A. This group was associated with reduced susceptibility to only a single antibiotic, cefoxitin, a 2nd generation cephalosporin. Only two *E. coli* group D isolates were found to exhibit phenotypic resistance in our study, but both were resistant to all cephalosporins tested. The eight resistant isolates in groups B1 and B2 were more heterogenous, exhibiting both single and multidrug resistance. These findings suggest a low chance of exposure to resistant pathogens via irrigation water and irrigated crops. Similar findings were recorded in two studies in Portugal with 3.6% β -lactam drug resistant *E. coli* retrieval from irrigation water, 5.3% retrieval from fresh vegetables in household farms (30) and a very low level of ESBL genes detected in *E. coli* from irrigation waters from urban farms in Manila, Philippines (40). A higher AMR occurrence was reported from irrigation water at a Swiss fresh produce farm where 22% of recovered *E. coli* were ESBL-producers (41) and on Ecuador farms where 58% of *E. coli* isolated from irrigation water had the phenotype (37). There is a report that β -lactam resistance is disseminating with colonization of wildlife populations (42), and this factor could explain geographical differences in ESBL-producing capacity among environmental *E. coli* in certain regions.

No overall influence of water type was revealed on AMR to cephalosporins. In a study conducted in the Netherlands, MDR *E. coli* from wastewater treatment plant effluents were disseminating to surface waters (43). In our study, we did not find a greater association with ESBL-producing *E. coli* and reclaimed wastewater sites compared to all other sites, perhaps because penicillin concentration did not differ drastically among water types tested in concurrent pharmaceutical level testing (44). Other investigators have postulated that antibiotic concentrations in treated municipal

effluents (reclaimed water) are not high enough to induce AMR in bacteria, and that other point sources of pollution (industrially polluted surface water, untreated hospital effluent and untreated municipal sewage) may be more likely to have sufficiently high concentrations to exert selective pressure for AMR development (19). Of note, however, is that the ESBL-producing isolate with the most genetic determinants (but not the most phenotypic resistance) was a B2 isolate from reclaimed water (Table 2).

β -lactam resistance is predominantly conferred by SHV, TEM and CTX-M β -lactamases. The former, encoded by *bla*_{CTX-M} genes that preferentially confer resistance to cefotaxime and ceftriaxone, have become predominant. The *bla*_{CTX-M-15} genotype was associated with presumptively pathogenic group B2 *E. coli* isolated from surface and reclaimed water in the Netherlands (17). Others have reported detection of the *bla*_{CTX-M} determinants in *E. coli* and other Enterobacteriaceae from irrigation water (41, 45). In our study *bla*_{CTX-M} was found in only 2 group B2 isolates from reclaimed water and one group D isolate from a tidal river, all three confirmed ESBL-producers, suggesting a low prevalence for this determinant in mid-Atlantic water available for irrigation of crops. Instead, *bla*_{CMY-2} encoding AmpC β -lactamase, was the most predominant gene detected, found in all group A and one-half of groups B1, B2 and D isolates that exhibited a resistant phenotype. The only other gene detected in all phylogroups was *bla*_{TEM}. However, the *bla*_{SHV} a combination that also more rarely confers ESBL activity, depending on the gene variants present, was only detected in one B2 isolate that also carried *bla*_{CTX-M}. The *bla*_{TEM} gene was also commonly detected in *E. coli* from both irrigation water and vegetables on household farms in Portugal (30). Group A and B1 isolates, noted above as groups less likely to associate with severe intestinal illness, carried fewer resistance determinants with a genotype characterized by *bla*_{CMY-2}, *bla*_{CMY-1} and *bla*_{TEM}. Group A isolates clustered together in hierarchical cluster analysis, reflecting the low phenotypic and gene carriage detected in these isolates. By contrast, group B2 and D isolates, noted above as more likely to associate with symptomatic and persistent diarrhea, were characterized by a heterogeneous β -lactamase genotype. Isolates in groups B2 and D clustered together with B1 isolates. The latter isolates were mixed in gene carriage status, ranging from no genes detected, to being positive to all genes tested. The dichotomy is interesting and should be explored further, especially in relation to crop growing season and times of highest irrigation frequency. The findings concurred with other reports that *bla*_{CMY} and/or *bla*_{TEM} were the most widespread genes in phylogroup A and B1, while *bla*_{CTX-M} more commonly associated with phylogroups B2 and D (20, 46).

Conclusion. Our study provides an integrated assessment of the phylogenetic analysis, and phenotypic and genotypic β -lactamase-producing traits of *E. coli* recovered from irrigation water sources in the mid-Atlantic United States. Phenotypic resistance to cephalosporins in *E. coli* was observed at a low prevalence (3.3%). Most phenotypic resistance and genetic determinants were identified in group B2 and D isolates from reclaimed water and rivers. Resistant isolates could be grouped into two main clusters. One group was composed primarily of phylogroup A isolates collected in summer and fall, exhibiting resistance to cefoxitin but susceptibility to cefotaxime, ceftriaxone, cefuroxime and ceftazidime. Another group comprised of phylogroup B1, B2 and D isolates with a tendency for MDR, retrieved in fall and winter samplings when vegetable crop production is low. These isolates were more likely to exhibit phenotypic resistance to cefotaxime, ceftriaxone, cefuroxime and ceftazidime. Overall, ESBL-producing *E. coli* were recovered at low prevalence in pond, rivers and reclaimed water examined. Small sample sizes for some water types and the restriction on reclaimed water sample collection in the winter were limitations of our study that should be considered in data interpretation. Nevertheless, resistance to β -lactams in reclaimed water warrants further investigation, as those isolates differed in their resistance traits. Findings from this study support the preferential use of pond water for irrigation of fresh produce crops over river water to minimize the dissemination of ESBL-producing *E. coli* via food. Understanding factors that favor ESBL producers in surface and reclaimed waters can inform decisions that support One Health.

TABLE 3 List of primers used for identification confirmation, classification and resistance gene detection in *E. coli* isolates

Gene	Size (bp)	Primer	Sequences (5'–3')	References/notes
<i>uidA</i>	192	Forward	CAGTCTGGATCGCGAAAA	27
		Reverse	ACCAGACGTTGCCACATA	
16S rRNA	357	Forward	AGAGTTTGATCCTGGCTCAG	47
		Reverse	TGACGGGCGGTGTGTACAAG	48
<i>chuA</i>	279	Forward	GACGAACCAACGGTCAGGAT	10
		Reverse	TGCCGCCAGTACCAAAGACA	
<i>yjaA</i>	211	Forward	TGAAGTGTGAGGAGACGCTG	
		Reverse	ATGGAGAATGCGTTCCTCAAC	
<i>TspE4.C2</i>	152	Forward	GAGTAATGTCGGGGCATTCA	
		Reverse	CGCGCCAACAAAGTATTACG	
<i>bla</i> _{CTX-M}	593	Forward	ATGTGCAGYACCAGTAARGT	49; designed from conserved regions of <i>bla</i> _{CTX-M} genes,
		Reverse	TGGGTRAARTARGTSACCAGA	including <i>bla</i> _{CTX-M-1} to <i>bla</i> _{CTX-M-30}
<i>bla</i> _{TEM}	964	Forward	GCGGAACCCCTATTTG	50
		Reverse	ACCAATGCTTAATCAGTGAG	
<i>bla</i> _{SHV}	854	Forward	TTCGCCTGTGATTATCTCCCTG	51
		Reverse	TTAGCGTTGCCAGTGYTCG	
<i>bla</i> _{CMY-1}	915	Forward	GTGGTGGATGCCAGCATCC	
		Reverse	GGTCGAGCCGGTCTTGTGAA	
<i>bla</i> _{CMY-2}	758	Forward	GCACTTAGCCACCTATACGGCAG	
		Reverse	GCTTTTCAAGAATGCGCCAGG	
<i>bla</i> _{OXA-1}	820	Forward	ATGAAAAACACAATACATATCAACTTCGC	50
		Reverse	GTGTGTTTGAATGGTGATCGCAT	
<i>bla</i> _{OXA-2}	602	Forward	ACGATAGTTGTGGCAGACGAAC	51
		Reverse	ATYCTGTTTGGCGTATCRATATTC	

MATERIALS AND METHODS

Sample collection, processing, and *E. coli* isolation. Water samples ($n = 333$) were collected from nine potential irrigation water sites and two on-farm irrigation ponds in the mid-Atlantic region between September 2016 and October 2018. The research was reviewed by the University of Maryland College Park Institutional Review Board (IRB, project number 964795-1) and was deemed exempt due to minimal risk to farm owners. Four different water types were included, non-tidal freshwater river/creek (site codes: MA03, MA04, MA05, MA07, MA09), tidal brackish river water (site code: MA08), pond water (site codes: MA10, MA11) and tertiary treated reclaimed wastewater (site codes: MA01, MA02, MA06). Water was collected according to the method described in Solaiman et al. (5). Within 12 h of sampling, standard membrane filtration was carried out according to U.S. EPA method 1604 (25). Briefly, water was filtered through 0.45 μ m, 0.47 mm nitrate ester membranes (Pall Corporation, Ann Arbor, MI, USA) and the filters incubated at 37°C for 24 h on MI agar (Becton, Dickinson and Company (BD), Franklin Lakes, NJ, USA). A total of 724 single presumptive *E. coli* colonies (maximum 3 colonies per sample) were isolated from the membranes and re-streaked onto MacConkey agar (BD) for further identification confirmation.

Since the sites included farms, the study was reviewed by the University of Maryland College Park Institutional Review Board (IRB) (project number 964795-1) and was approved as exempt due to minimal risk to farm owners.

DNA extraction and identification of *E. coli* by PCR. For PCR confirmation of presumptive *E. coli* ($n = 724$), colonies from MacConkey agar were resuspended in 7.5% sterile Chelex100 (Sigma-Aldrich, St. Louis, MO, USA) solution for DNA extraction using a rapid heat lysis method (26). A species-specific PCR (PCR) was employed by amplifying the β -glucuronidase gene, *uidA*, to confirm identification of *E. coli* isolates (27). Amplification was performed in 1X PCR buffer (New England Biolabs [NEB], Ipswich, MA, USA), 1.5 mM MgCl₂ (NEB), 0.2 mM dNTPs (NEB), 0.4 μ M 16S rRNA gene primers (internal control) and *uidA* primers (Integrated DNA Technologies (IDT), Coralville, IA, USA) (Table 3), 5 Units *Taq* DNA polymerase (NEB) and ~20 ng of template DNA. The standard cycling conditions consisted of an initial single cycle at 95°C for 30 s, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 30 s and a final single cycle at 72°C for 5 min. *E. coli* CFT073-UPEC was used as a positive control.

***E. coli* phylogrouping.** Phylogrouping of *E. coli* isolates ($n = 724$) was performed using a triplex PCR by amplification of the three virulence genes: heme transport gene (*chuA*), stress related gene (*yjaA*) and lipase/esterase gene fragment (*TspE4C2*), adapted from Clermont et al. (10). The multiplex amplification was performed in 1X PCR buffer (NEB), 1.5 mM MgCl₂ (NEB), 0.2 mM dNTPs (NEB), 0.4 μ M *chuA*, *yjaA* and *TspE4C2* primer pairs (IDT) (Table 3) and 5 units *Taq* DNA polymerase (NEB). Approximately 20 ng of template DNA was used for the reaction. The standard cycling conditions consisted of an initial step at 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, elongation at 72°C for 30 s, followed by a final elongation at 72°C for 5 min. Amplified products were separated by electrophoresis on 2% agarose gel (Lonza, Rockland, ME, USA). *E. coli* CFT073-UPEC was used as a positive control.

Phenotypic determination of cephalosporin resistance in *E. coli*. Each *E. coli* isolate ($n = 724$) was inoculated on Tryptic Soy Agar (TSA; BD), subcultured in Mueller-Hinton broth (MHB) (BD) and incubated at 37°C with shaking for 3 h. Cultures were spread plated onto pre-prepared Mueller-Hinton agar plates (MHA) (BD). Five cephalosporin disks including 2nd generation cefoxitin (30 µg; FOX30) and cefuroxime (30 µg; CXM30), and 3rd generation ceftazidime (30 µg; CRO30), cefotaxime (30 µg; CTX30) and ceftazidime (30 µg; CAZ30) were placed on MHA plates. Plates were incubated at 37°C for 18–20 h according to standard recommendations (28). Data were interpreted from the diameter of the inhibition zone according to CLSI (28).

Determination of ESBL activity. Sixteen isolates that were positive for either ceftazidime or cefotaxime resistance were further tested for ESBL activity by disk diffusion according to CLSI recommendations (28). Suspensions at 0.5 MacFarland were made in sterile water from fresh 20-h MH agar cultures and spread on fresh MH agar. Antibiotic disks (ceftazidime 30 µg, ceftazidime-clavulanic acid 30/10 µg [BD]), cefotaxime 30 µg (Hardy Diagnostics, Santa Maria, CA, USA) and cefotaxime-clavulanic acid 30/10 µg (BD) were aseptically placed onto plates and incubated at 35°C for 17 h. The difference in the zone of inhibition between disks supplemented with clavulanic acid and disks with antibiotic alone was recorded and isolates with a difference of ≥ 5 mm were considered ESBL-producing.

Genotypic evaluation of β -lactam resistance in *E. coli*. This analysis was performed on isolates that exhibited phenotypic AMR to at least one antibiotic. Three sets of multiplex PCR amplifications were employed for detection of the β -lactam antimicrobial resistance genes bla_{TEM} , bla_{SHV} , bla_{CTX-M} , bla_{CMY-1} , bla_{CMY-2} , bla_{OXA-1} and bla_{OXA-2} . Primer pairs used for detection of these genes are listed in Table 3. DNA was extracted from resistant isolates identified through phenotypic testing using the Qiagen DNeasy Ultraclean Microbial DNA Extraction Kit (Qiagen, Hilden, Germany). Multiplex PCR was done with 1X PCR buffer (NEB), 1.5 mM MgCl₂ (NEB), 0.2 mM dNTPs (NEB) and 0.3 µM each primer (IDT). Standard cycling condition for amplification were performed using 3 protocols. Protocol I included an initial step (95°C for 15 min) followed by 30 cycles of amplification, 95°C for 60 s, 55°C for 40 s, and 72°C for 60 s, followed by a final elongation at 72°C for 3 min for bla_{TEM} - bla_{SHV} -16S rRNA genes. For protocol II, standard cycling conditions started with an initial step at 95°C for 15 s, followed by 30 cycles of amplification comprised of 95°C for 60 s, 60°C for 40 s, 72°C for 60 s, with a final elongation step at 72°C for 3 min for bla_{CMY-1} - bla_{CMY-2} - bla_{OXA-1} - bla_{OXA-2} -16S rRNA genes. For protocol III, standard cycling conditions started with an initial step at 95°C for 1 min followed by 30 cycles of amplification comprised of 95°C for 15 s, 52°C for 15 s, 72°C for 10 s for bla_{CTX-M} -16S rRNA genes. PCR products were resolved on 2% agarose (Lonza) gels to determine presence or absence of target genes.

Data management and statistical analysis. To avoid clonality, 488 out of 724 *E. coli* isolates were selected for data analysis based on unique combinations of water source, collection date, phylogroup assignment, phenotypic AMR profile and resistance gene carriage. Data were pooled by water type (five freshwater river, one tidal river, two ponds, and three reclaimed water sites) and season (from 1 March to 30 May as spring, from 1 June to 31 August as summer, from 1 September to 30 November as fall, from 1 December to 28 February as winter) as in Solaiman et al. (5). No reclaimed water samples were collected in the winter. Results for analysis were verified by rerunning analysis excluding reclaimed water or winter data. Phylogroup evenness was calculated for each water type using Shannon's Equitability Index (E_H):

$$E_H = - \frac{\sum_i \left(\frac{n_i}{N} \cdot \ln \left(\frac{n_i}{N} \right) \right)}{\ln N}$$

where n is the number of isolates per phylogroup, i is the number of phylogroups, N is the total number of isolates. Isolates were grouped according to resistance to a single cephalosporin drug (SDR) or two or more cephalosporins (MDR). Effect of variables was assessed using the χ^2 -squared test with $\alpha = 0.05$. Multinomial logistic regression was used to assess the effect of season or water type on *E. coli* phylogroup distribution. Nominal regression was used to assess group distribution by site. Hierarchical cluster analysis (HCA) using the group average method was conducted to construct a dendrogram from a similarity matrix using the simple matching coefficient based on phenotypic resistance and the presence/absence of β -lactam resistance genes among the four *E. coli* phylogroups. The simple matching coefficient was selected as it resembles the Jaccard index but also counts both mutual presence and mutual absence of attributes between samples. ANOSIM was used to evaluate the similarity between groups, phylogroup, water type and season. Multiple correspondence analysis (MCA) was conducted using group classification, presence/absence of resistance genes, season and water type to assess the associations among these categorical variables. Statistical analysis and graphical representation were performed in R ver. 3.6.3. HCA and ANOSIM were conducted in Primer 6 ver. 6.1.15 and logistic regression and MCA analysis were performed in JMP Pro ver. 15.2.0.

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