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TECHNICAL REPORT

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Lack of wastewater treatment in a small town drives the spread of ESBL-producing *Escherichia coli* in irrigation waters

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ABSTRACT

Antibiotic resistance (ABR) is a critical and growing global challenge, especially in low- and middle-income countries. Ecuador has made great progress in connecting households to piped water supplies; however, the collection and treatment of domestic wastewater has lagged. This infrastructural gap may be accelerating the spread of ABR into surface waters used downstream for irrigation. We studied the contributions of a small town in Ecuador to the prevalence of extended-spectrum β-lactamase-producing *E*scherichia *coli* in a glacial stream used for irrigating crops. The study analyzed water samples upstream ($n = 60$) and downstream ($n = 60$) of the town of Píntag as well as 30 lettuce samples irrigated by surface waters downstream of the town. A subset of third generation cephalosporin resistant *E. coli* (3GCR-EC) isolates ($n = 58$) were sequenced to characterize antibiotic resistance genes and pathogenic lineages. Our results showed that there was nearly a three-log increase in mean *E. coli* colony forming units in the downstream samples versus upstream. At the upstream sites above the town of Píntag, 6.7% of water samples were positive for 3GCR-EC compared to 100% of samples collected at the downstream sites. Additionally, 70.1% of sequenced 3GCR-EC isolates collected at downstream sites carried $bla_{\text{CTX-M}}$ genes and 3.4% belonged to pandemic lineages ST131 and ST10. As countries develop household piped water infrastructure, attention should focus on how the lack of domestic wastewater collection and treatment may accelerate the spread of ABR in waterways and the food system.

Plain Language Summary

Small towns without wastewater treatment release raw sewage, potentially containing antibiotic-resistant bacteria. This sewage contaminates water used to irrigate edible crops like lettuce. In this study, we measured antibiotic-resistant bacteria in water

Abbreviations: 3GCR-EC, third generation cephalosporin resistant *E. coli*; ABR, antibiotic resistance; AM, ampicillin; AMC, amoxicillin-clavulanate; ARG, antibiotic resistance gene; CAZ, ceftazidime; CFU, colony forming unit; CTX, cefotaxime; CZ, cefazolin; ESBL, extended-spectrum β-lactamase; ESBL-EC, extended-spectrum β-lactamase *E. coli*; LMICs, low- and middle-income countries; MDR, multidrug resistance; ST, sequence type; TE, tetracycline.

upstream and downstream of a small town in Ecuador that lacks wastewater collection and treatment. We also measured antibiotic-resistant bacteria on lettuce grown irrigated with water downstream of the small town. We found that water downstream of the small town contained a much higher level of antibiotic-resistant bacteria than samples collected upstream of the town. We also found high numbers of antibiotic-resistant bacteria on the sampled lettuce. This study highlights the importance of wastewater collection and treatment to prevent the spread of antibiotic-resistant bacteria.

1 | INTRODUCTION

The global burden of antibiotic-resistant infections caused by multidrug-resistant *Escherichia coli* is growing rapidly in prevalence around the world (Pulingam et al., 2022). The threat of antibiotic resistance (ABR) has important implications for population health as it undermines the treatment of life-threatening bacterial infections. In 2019, global estimates attributed 1.2 million deaths to ABR, with this number projected to rise to 10 million by 2050 if actions to combat the issue are not taken (O'Neill, 2016). Extended-spectrum β-lactamase *E. coli* (ESBL-EC) are of particular concern because of their ability to confer resistance to multiple classes of antibiotics (Montero et al., 2021; Murray et al., 2022). Pathogenic strains of ESBL-EC cause a range of drug-resistant infections such as urinary tract infections, blood stream infections, meningitis, and hemorrhagic colitis, which may ultimately result in death (Mellata, 2013).

The rise in resistance to third-generation cephalosporin (3GC) antibiotics, essential for addressing infections caused by both Gram-negative and Gram-positive bacteria, is linked to mechanisms involving extended-spectrum β-lactamases (ESBLs) (Arumugham et al., 2024; Mark et al., 2021). ESBL genes are increasingly reported globally and have been noted for their potential to have epidemic potential (Castanheira et al., 2021). In February 2024, the World Health Organization reevaluated the significance of medically important antimicrobials, designating all 3GCs as "High Priority Critically Important Antimicrobials" (HPCIAs), underscoring their pivotal role in human health and the imperative to safeguard their efficacy amidst mounting ABR trends (WHO, 2024).

The adoption of a One Health approach can help address the role played by the environment in the spread of ABR, which is especially relevant in low- and middle-income countries (LMICs) (Lincopan et al., 2023). The contamination of streams with wastewater is likely to increase as urbanization continues and households gain access to piped water connections (Sato et al., 2013). Additionally, droughts exacerbated by climate change may stress the already scarce quantities of clean irrigation water in LMICs, forcing more and more regions to change their water sources to untreated wastewater as an adaptation measure (Al Hamedi et al., 2023).

Ecuador is categorized as an upper middle-income nation and has an economy heavily reliant on agriculture. The country faces a challenge concerning the lack of wastewater treatment plants and the utilization of wastewater-contaminated water for irrigation purposes. The disposal of waste into the environment untreated is common practice, which eventually runs off into rivers and streams that are subsequently utilized for irrigation by downstream farms (Egas & Ordoñez, 2010). Although irrigation channels are often designed to limit fecal contamination, cross-contamination can occur (Kümmerer, 2003). Ecuador has managed to make great progress in delivering piped potable water to its citizens, yet the infrastructure needed to treat the domestic wastewater is still underdeveloped. It is estimated that only 31% of domestic wastewater in Ecuador is treated, constituting one of the lowest percentages for the Latin America and the Caribbean region (World Bank, 2017). A 2021 study on surface water and produce quality across 17 provinces in Ecuador revealed that a significant portion of ESBL-producing isolates originated from irrigation water (58%). Notably, all irrigation water samples tested positive for ESBL-EC, underscoring the importance of irrigation water as a reservoir and pathway of exposure to ABR (Montero et al., 2021). This contamination poses a notable health risk due to the introduction of ESBL-EC into the food chain through contaminated waterways, thereby potentially exacerbating human exposures (Montero et al., 2021). Of particular concern is the consumption of produce, including leafy greens, which have been linked to multi-drug-resistant *E. coli* outbreaks due to fecal contamination (Priyanka et al., 2021), and contaminated irrigation water has been suggested to be a primary contributor to the contamination of fresh produce with various other pathogenic microorganisms (Njage & Buys, 2017).

This study aimed to measure the microbiological quality, defined as the quantity of ESBL-EC bacteria and associated resistance genes, of a watershed upstream and downstream of a small rural town. We aimed to understand how the absence of wastewater treatment affected water quality and

ABR presence, as well as understand how this affected the microbiological quality of produce. This study contributes to our understanding of how the lack of wastewater collection and treatment can result in contaminated irrigation water that augments the spread of ABR through the food chain (M. Amato et al., 2021; Priyanka et al., 2021).

Core Ideas

- Lack of domestic wastewater treatment negatively impacts irrigation water quality.
- Irrigation water harbors diverse extended-spectrum beta-lactamase producing bacteria that can potentially end up on produce.
- Genomic analyses offer insights about potential health risks from enteric pathogens and antibiotic resistance genes entering irrigation waters.
- Effective wastewater management is essential for reducing the spread of antibiotic resistance in the human-animal-environment interface, especially where piped water exists but no wastewater collection and treatment occurs.

2 | MATERIALS AND METHODS

2.1 | Study area

The study was conducted in a watershed within the Pita River basin, southeast of Quito, Ecuador. A stream formed from the Sincholagua and Antisana volcanoes flows down the watershed from the highlands, eventually passing through the small town of Píntag (pop. ~23,000). This watershed was delineated using ESRI's ArcGIS Pro with a detailed digital elevation model for the Pichincha province. Water from the stream is used for irrigation in surrounding farms. The local Píntag market in the town center was visited for purchasing lettuce irrigated with water from this same stream. This region was chosen due to its well-defined watershed boundaries (Figure 1), the presence of locally raised crops irrigated by a stream that has minimal sources of contamination, except for the town of Píntag, which lacks wastewater collection and treatment, shown in Figure S1.

2.2 | Water sampling

Sampling was conducted at four total water sites: two presumed high-quality sites (HQ1 and HQ2) approximately 3 km away from each other upstream near the top of the watershed, and two presumed low-quality sites (LQ1 and LQ2) approximately 1 km from each other and within 1 km downstream of the Píntag town borders. At the upstream sample sites, 100 mL Aquagenx gel bags containing ceftriaxone (1 mg/L) were used to collect water samples and culture third generation cephalosporin resistant *E. coli* (3GCR-EC). Additionally, 100 mL Aquagenx gel bags containing 90 mL purified, autoclaved water (1:10 dilution of stream water) were used to collect stream water to quantify *E. coli*. Similarly, at downstream samples sites, 100 mL Aquagenx gel bags containing ceftriaxone (1 mg/L) were used for detecting 3GCR-EC. Additionally, 100-mL gel bags containing 99 mL purified water were used to collect irrigation water at a (1:100 dilution of stream water). Note, a higher dilution was used downstream because colonies were too numerous to count at the 1:10 dilution. The gel bags were prepared on a flat surface to solidify and then placed in a cooler and transported to the Microbiology Institute at Universidad San Francisco de Quito for incubation within 4 h of collection. Sample sites were tested once each week for 3 weeks for a total of 120 water samples collected across all sites. A negative control (100 mL of filtered, autoclaved water) was used each week during sample collection. Coordinates of these sites and detailed collection information can be found in Table S1.

2.3 | Produce sampling and analysis

Unwashed heads of lettuce (*Lactuca sativa)* were collected at the central market in Píntag once a week for 2 weeks that overlapped with water sampling. Farmers vending their produce verbally confirmed whether their lettuce was irrigated with the sampled stream. Only lettuce irrigated with water from the stream of interest was purchased for sampling. Aquagenx field kits were used to measure *E. coli* isolates from lettuce and quantify colony forming units (CFUs), following manufacturer guidelines. Using aseptic methods, two 10 g samples of lettuce were taken from a lettuce head and each 10 g sample was placed in 100 mL of filtered, autoclaved water within a whirlpack bag and shaken vigorously for 20 s. Note that 100 mL of the lettuce wash was then carefully poured into Aquagenx field kits; one lettuce wash sample cultured with ceftriaxone (1 mg/L concentration) and one lettuce wash sample cultured without ceftriaxone. This method was repeated for 30 lettuce samples ($n = 60$ leaves) during the same weeks as water sampling. Two negative batch controls were taken for each processing day. Further details regarding lettuce sampling and processing can be found in Table S1.

2.4 | Sample processing and bacterial culture

Aquagenx gel bags containing the samples were incubated at 35–37°C for 20 h. The EC growth media is a chromogenic agar containing substrates with the ability to detect

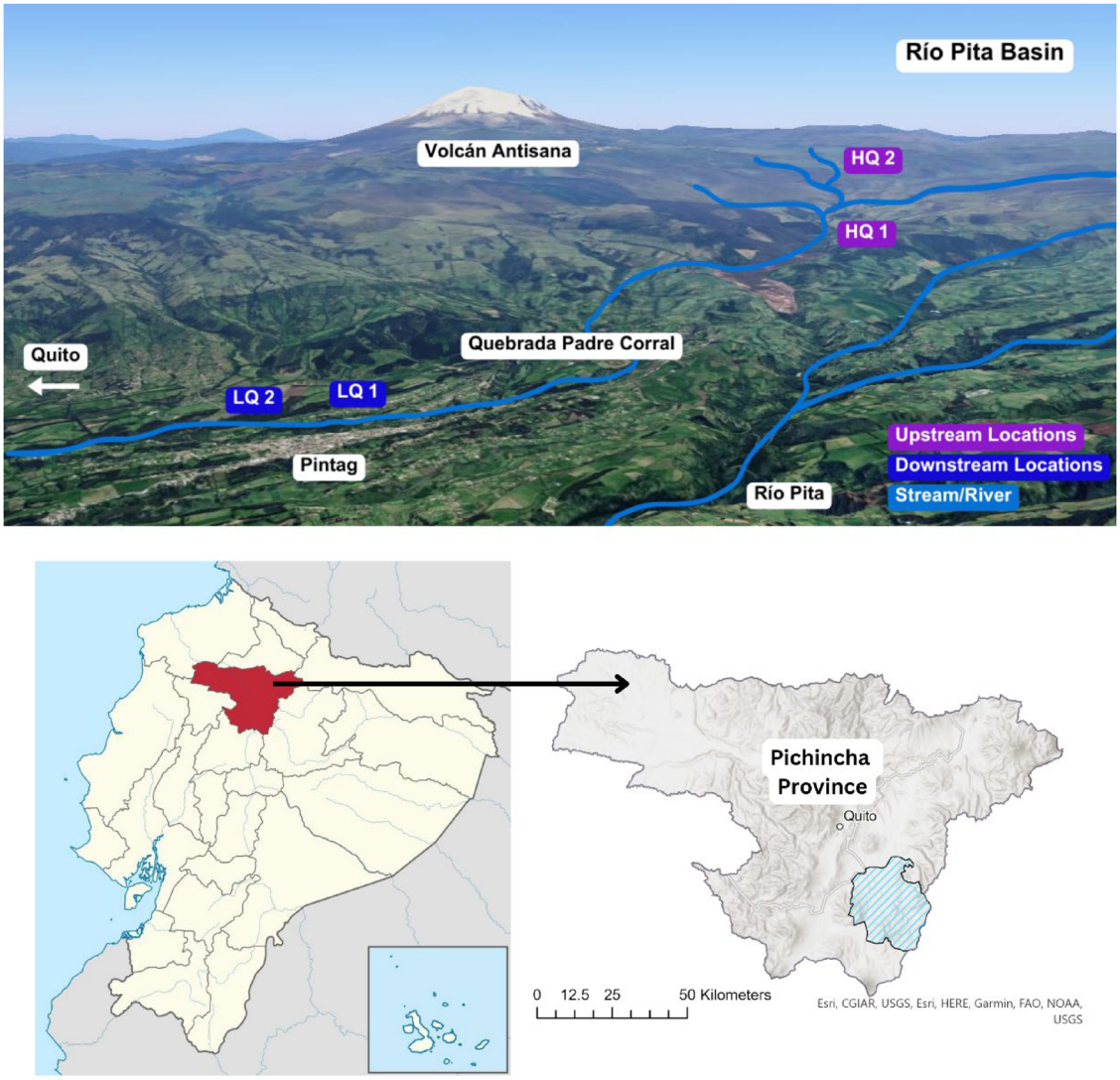

FIGURE 1 Two upstream (HQ = high quality) and two downstream sampling sites (LQ = low quality) where water samples were collected within the Pita River Basin, Southeast of Quito, Ecuador.

β-glucuronidase, an enzyme produced by most strains of *E. coli* (Burnet et al., 2019). When *E. coli* colonies metabolize the media, the colonies appear blue in color.

Following incubation, photos of each gel bag were captured, and clearly visible colonies were counted by hand in ambient light. The method of counting colonies remained consistent throughout the study and did not differ between water or lettuce samples or whether they contained ceftriaxone. Two random colonies were extracted from the Aquagenx gels (gels with and without ceftriaxone) using sterile, disposable syringes. To do this, the front and back of the gel bag was first sterilized using lab-grade isopropyl alcohol and allowed to dry. Then, using a disposable syringe, the bag was pierced at the site of the randomly selected colony, extracting the colony by the tip of the needle, and plated onto MacConkey agar. A maximum of two colonies were extracted from a single sample. If a gel bag contained one colony, only the single colony would be extracted and plated. Samples from bags containing ceftriaxone were plated onto ceftriaxone-supplemented MacConkey agar (1 μg/mL) to ensure only putative *E. coli* resistant to the antibiotic would grow. The plates were incubated at 35–37˚C for 20–24 h.

After incubation, two single random colonies were carefully extracted from the MacConkey agar plates and placed into 2-mL tubes of semi-solid tryptic soy broth. The tubes were incubated overnight at 35–37˚C and placed in the refrigerator and stored for further testing.

2.5 | Antibiotic susceptibility testing

Antibiotic susceptibility testing was conducted for 75 randomly selected isolates from high-quality water (n = 28), low-quality water (n = 31), and produce (n = 15). The

isolates were tested against 12 antibiotics using the Kirby Bauer Disk Diffusion method on Mueller–Hinton agar. All isolates were randomly selected from their sample cultures and were from samples not selectively cultured in the presence of ceftriaxone. Susceptibility testing included amoxicillin-clavulanate [AMC, 20/10 ϵg], ampicillin [AM, 10 ϵg], Cefazolin [CZ; 30 ϵg], Ceftazidime [CAZ; 30 µg], Cefotaxime [CTX; 30 µg], Cefepime [FEP; 30 µg], Chloramphenicol [C; 30 ϵg], Ciprofloxacin [CIP; 5 ϵg], Gentamicin [GM; 10 ϵg], Imipenem [IPM; 10 ϵg], Tetracycline [TE; 30 ϵg], and trimethoprim-sulfamethoxazole [SXT; 1.25/23.75 ϵg]). The zones where bacterial growth was inhibited around each antibiotic were measured with a caliber tool in millimeters and recorded. Using breakpoints from the Clinical and Laboratory Standards Institute (CLSI, 2018), each isolate was classified as either susceptible, intermediate, or resistant. Presumptive ESBL-producing isolates were based on their phenotypic resistance to AM in addition to CAZ, CTX, or AMC (Kumar et al., 2014).

2.6 | DNA extraction and genomic analysis

E. coli DNA extraction was conducted using Qiagen DNeasy Blood & Tissue Kits for 48 ceftriaxone-resistant *E. coli* isolates, 30 from low-quality water samples and 18 from produce samples. An additional 12 putative *E. coli* from lettuce samples that grew on MacConkey agar without ceftriaxone were also included. Whole genome sequencing data were generated using Illumina short read, paired-end protocol. The quality of the reads was assessed using FastQC v0.12.1 (Wingett & Andrews, 2018). De novo assemblies of the paired short reads were generated using the SPAdes optimizer tool Unicycler c.0.3.0b (Wick et al., 2017). Contigs below 500 bp were excluded from final draft assemblies. QUAST v5.0 (Mikheenko et al., 2018) was used to assess the quality of assembled sequences. Sequence types (STs) of the assembled isolate genomes were determined by MLST version 2.19 (Seemann, n.d.-b). Resistance genes were annotated using the ResFinder database of the ABRicate tool v.1.0 (Seemann, n.d.-a), with a threshold of 80% coverage and 80% match.

3 | RESULTS

3.1 | Prevalence of *E. coli* in water and lettuce samples

A total of 180 samples were collected over the course of 3 weeks: 60 from upstream water sites, 60 from downstream sites, and 60 from lettuce samples. All downstream water samples were positive for *E. coli*, including samples cultured in the presence of ceftriaxone. In upstream samples, two samples were positive for those cultured in the presence of ceftriaxone (6.7%), and all the upstream samples without ceftriaxone were positive for *E. coli* (Table 1).

3.2 | Antibiotic susceptibility testing

Phenotypic resistance to 12 antibiotics was evaluated among the 75 *E. coli* isolates, none of which were cultured in the presence of ceftriaxone. Out of these, 16 isolates exhibited resistance to at least two antibiotics (21%), while seven displayed resistances to three or more classes (16%), meeting the criteria for multidrug resistance (MDR). For subgroup analysis, the study comprised 29 random isolates from upstream locations, 31 from downstream sites, and 15 isolates from produce (Table 2). None of the upstream isolates demonstrated MDR, with only one isolate showing resistance to two antibiotics (CZ and AM). In contrast, downstream isolates exhibited a higher prevalence of MDR, with 22.5% demonstrating the MDR phenotype (Figure 2). In addition, 86.6% of the lettuce isolates were resistant to at least one antibiotic, all but one of which included resistance to CZ.

A total of 19 *E. coli* isolates (25%) displayed the ESBL phenotype. Downstream isolates constituted the largest proportion of the ESBL phenotype ($n = 9$), followed by lettuce ($n = 7$), and upstream isolates ($n = 3$), though these were not found to be statistically different. Forty-six percent of lettuce isolates and 23% of downstream isolates demonstrated the ESBL phenotype. Among all the water samples, AM, CZ, and TE resistance was identified in both up and downstream water samples.

3.3 | Antibiotic resistance genes (ARG)

Among 48 isolates that were sequenced, 26 harbored a $bla_{\text{CTX-M}}$ gene, the most common allelic variant being $bla_{\text{CTX-M-65}}$, found in 29% of downstream isolates. The $bla_{\text{CTX-M-14b}}$ gene was identified in six isolates, $bla_{\text{CTX-M-8}}$ in four isolates, and $bla_{\text{CTX-M-55}}$ was found in two isolates. Analysis of sequenced isolates revealed a high prevalence of beta-lactam resistance genes, with 91.7% of all sequenced isolates carrying at least one beta-lactam gene (Figure 3). Forty-two percent of isolates harbored two beta-lactam genes (Figure 4), with the predominant combination being a bla_{TEM}-type gene paired with a bla_{CTX}-type gene.

The study identified one carbapenem resistance gene, $bla_{\text{OXA-427}}$, in a lettuce sample where the bacterium was an unknown species.

Among the *E. coli* isolates sequenced, a range of STs existed (Figure 5), and 34 STs were identified overall. The most frequent ST found was ST162 (32.3% of isolates),

TABLE 1 Mean *Escherichia coli* colony forming units (CFUs) per 100 mL of sampled water from upstream and downstream sampling sites.

	Stream samples cultured with ceftriaxone supplement (1 mg/1L)	Stream samples
Upstream sites ($n = 60$) Mean CFU (standard deviation [SD])	0.3 (±1.6)	52.2 (±35.1)
Downstream sites ($n = 60$) Mean CFU (SD)	2596.7 (±1960.6)	27,940.0 (±18,321.7)
p-value (t-test)	3.3E-08	5.5E-09

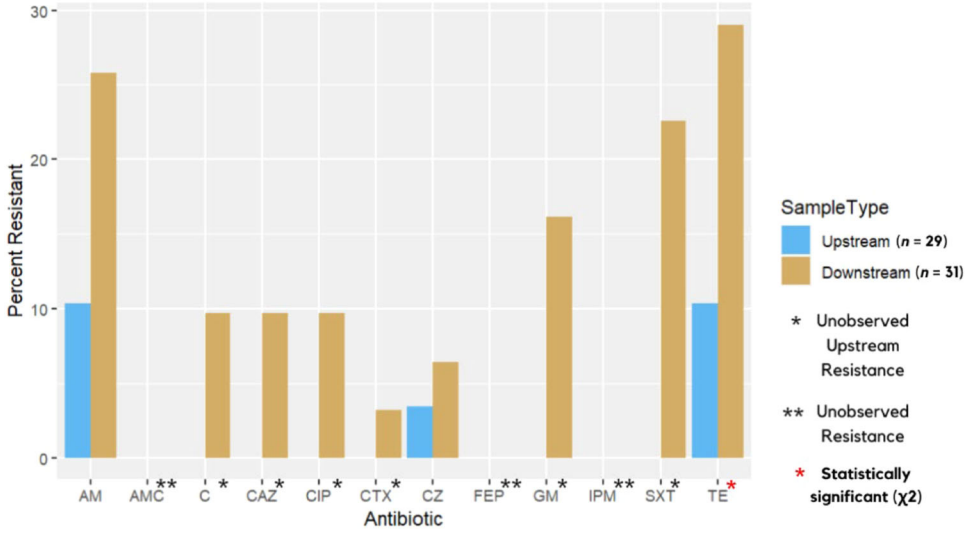

FIGURE 2 Phenotypic antibiotic resistance for 75 *Escherichia coli* isolates sampled from irrigation water upstream and downstream of Píntag, Ecuador. Antibiotics included ampicillin (AM; 10 εg), amoxicillin-clavulanate (AMC; 20/10 εg), chloramphenicol (C; 30 εg), ceftazidime(CAZ; 30 εg), ciprofloxacin (CIP; 5 εg), cefotaxime (CTX; 30 εg), cefazolin (CZ; 30 εg), cefepime (FEP; 30 εg), gentamicin (GM; 10 εg), imipenem (IPM; 10 εg), trimethoprim-sulfamethoxazole (SXT; 1.25/23.75 εg), and tetracycline (TE; 30 εg).

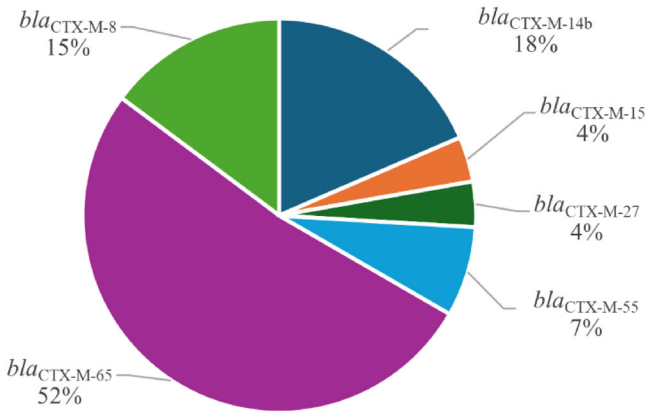

FIGURE 3 Distribution of bla_{CTX-M} genes from downstream irrigation water and lettuce *Escherichia coli* isolates ($n = 27$).

followed by ST38, ST1706, and ST48. We found no overlap of STs among water and lettuce samples. Pandemic lineage STs ST131 and ST10 were identified and were also found to harbor ESBL genes. A summary table of the results, including the ST and β-lactamase genes, identified in each isolate sequenced can be found in Table S2.

4 | DISCUSSION

The findings of this research highlight the impact of one small town lacking wastewater collection and treatment on the quality of irrigation water within a watershed. The presence of ESBL-EC in irrigation water and on produce has implications for the food system, locally and potentially abroad. The stark contrast in *E. coli* CFU counts between upstream and downstream locations (mean of 0.3 CFU per 100 mL vs. mean of 52.2 CFU per 100 mL, p-value = 3.3E-08) within the watershed underscores the substantial influence of human activities on the prevalence of resistance to third generation cephalosporins. In comparing CFU counts, we found a dramatic increase in phenotypic resistance to antibiotics and a high number of *E. coli* positive for ESBL genes in downstream irrigation water. This is consistent with previous research, which found increasing CFU counts

TABLE 2 Phenotypic antibiotic resistance among randomly selected *Escherichia coli* from upstream water, downstream water, and produce.

		Upstream isolates ($n = 29$)			Downstream isolates ($n = 31$)			Produce isolates ($n = 15$)		
Drug class	**Type**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**
Penicillins	**Amoxicillin-clavulanate**	**0 (0%)**	**3 (10.3%)**	**26 (89.7%)**	**0%**	**2 (6.5%)**	**29 (93.5%)**	**6 (40%)**	**1 (6.7%)**	**8 (53.3%)**
	Ampicillin	**3 (10.3%)**	**1 (3.5%)**	**25 (86.2%)**	**8 (25.8%)**	**3 (9.7%)**	**20 (64.5%)**	**6 (40%)**	**2 (13.3%)**	**7 (46.7%)**
Cephalosporins	**Cefazoline (1G)**	**1 (3.5%)**	**7 (24.1%)**	**21 (72.4%)**	**2 (6.5%)**	**5 (16.1%)**	**24 (77.4%)**	**12 (80%)**	**0%**	**3 (20%)**
	Ceftazidime (3G)	**0%%**	**0%**	**100%**	**3 (9.7%)**	**0%**	**28 (90.3%)**	**1 (6.7%)**	**0%**	**14 (93.3%)**
	Cefotaxime (3G)	**0%**	**0%**	**100%**	**1 (3.2%)**	**1 (3.2%)**	**29 (93.5%)**	**1 (6.7%)**	**0%**	**14 (93.3%)**
	Cefepime (4G)	**0%**	**0%**	**100%**	**0%**	**0%**	**100%**	**1 (6.7%)**	**0%**	**14 (93.3%)**
Fluoroquinolones	**Ciprofloxacin**	**0%**	**0%**	**100%**	**3 (9.7%)**	**2 (6.5%)**	**26 (83.9%)**	**1 (6.7%)**	**0%**	**14 (93.3%)**
Aminoglycosides	**Gentamicin**	**0%**	**0%**	**100%**	**5 (16.1%)**	**0%**	**26 (83.9%)**	**1 (6.7%)**	**0%**	**14 (93.3%)**
Carbapenems	**Imipenem**	**0%**	**0%**	**100%**	**0%**	**0%**	**100%**	**0%**	**0%**	**100%**
Tetracyclines	**Tetracycline**	**3 (10.3%)**	**0**	**26 (89.7%)**	**9 (29%)**	**0%**	**22 (71%)**	**1 (6.7%)**	**0%**	**14 (93.3%)**
Folate Pathway Inhibitors	**Trimethoprim-sulfamethoxazole**	**0%**	**0%**	**100%**	**7 (22.6%)**	**0%**	**24 (77.4%)**	**0%**	**0%**	**100%**
Protein Synthesis Inhibitors	**Chloramphenicol**	**0%**	**0%**	**100%**	**3 (9.7%)**	**0%**	**28 (90.3)**	**0%**	**0%**	**100%**
Multidrug Resistant		**0%**			**7 (46.7%)**			**0%**		

Note: Phenotypic testing only used *Escherichia coli* cultured in the absence of ceftriaxone supplement.

Abbreviations: 1G, 1st generation cephalosporin; 3G, 3rd generation cephalosporin; 4G, 4th generation cephalosporin; I, intermediate; R, resistant; S, susceptible.

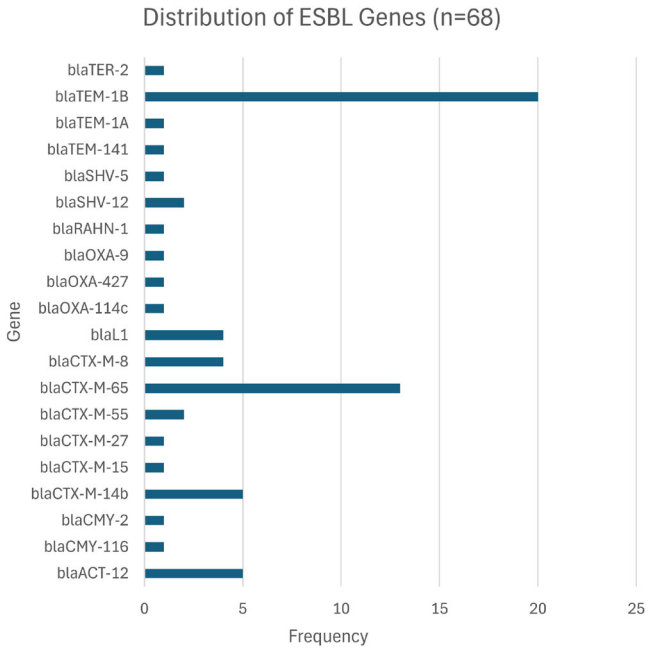

FIGURE 4 Bar chart displaying the counts of β-lactam-coding genes for the 48 successfully sequenced isolates.

correlated with an increased proportion of drug-resistant *E. coli* positive samples in irrigation water (Gekenidis et al., 2018).

The high prevalence of bacteria carrying $bla_{\text{CTX-M}}$ type genes in irrigation water downstream from Píntag is alarming due to their potential to spread to pathogenic bacteria (Cantón et al., 2012). A $bla_{\text{CTX-M}}$-type gene was discovered in 72.2% of *E. coli* that were cultured in the presence of ceftriaxone (26 of 36 isolates). The allelic variant $bla_{\text{CTX-M-65}}$ was found in 29% of downstream irrigation samples and is commonly isolated from *E. coli* from both humans and food-producing animals (Valenzuela et al., 2023). A 2023 study also found a high prevalence of the $bla_{\text{CTX-M-65}}$ gene isolated from fecal samples in children also from the Pichincha province of Ecuador (H. K. Amato et al., 2023). The second most prevalent allelic variant was $bla_{\text{CTX-M-14b}}$ (14.7%), followed by $bla_{\text{CTX-M-8}}$ (11.8%) and $bla_{\text{CTX-M-55}}$ (5.9%). These variants are consistent with studies of *E. coli* isolated from children, chickens, and dogs in a previous study where the alleles with the highest prevalence were $bla_{\text{CTX-M-55}}$ followed by $bla_{\text{CTX-M-65}}$ (Salinas et al., 2021). Additionally, $bla_{\text{CTX-M-8}}$ was found in *E. coli* from both downstream water and lettuce samples, suggesting that water and produce may be linked, although the STs of the *E. coli* differed. The results of this study suggest that some ARGs are good indicators of antibiotic use (e.g., ARGs coding resistance to 3rth generation cephalosporins or amoxicillin-clavulanate) whereas other ARGs aren't (e.g., ARGs coding resistance to tetracyclines and ampicillin).

In certain isolates, the resistance gene $bla_{\text{TEM-1b}}$ was found in potential pathogenic STs such as ST131 (Johnson et al., 2010). ST162 was the most prevalent ST, discovered in 11 of the 48 sequenced genomes (23%), all from downstream irrigation water samples. One hundred percent of the samples with this ST were associated with the $bla_{\text{CTX-M-65}}$ gene and 9 of the 11 were combined with the $bla_{\text{TEM-1b}}$ gene (81.2%). When compared to phenotypic results from antibiotic susceptibility testing, downstream ST162 isolates carrying the combined $bla_{\text{CTX-M-65}}$ and $bla_{\text{TEM-1b}}$ genes were more likely to be multidrug resistant compared to downstream samples without this S. ST162 appears in South American hospitals but has also been identified in environmental reservoirs. ST162 has been associated with ESBL-EC from livestock and in Andean condors (Fuentes-Castillo et al., 2020). In a study examining *E. coli* from ready-to-eat street food in Quito, ST162 was the most prevalent ST, illuminating a potential transmission pathway from contaminated irrigation water as a source (Zurita et al., 2020). ST162 has also previously been isolated from Ecuadorian rivers (Ortega-Paredes et al., 2019) and identified in patients with diarrhea and other infections (Zhang et al., 2021).

The significant impact to water quality from a small town without wastewater collection and treatment highlights the importance of the environment in the transmission of ARGs and the need for effective wastewater management practices in line with global development objectives. The United Nations Sustainable Development Goal 6.3, which aims to achieve universal wastewater treatment by 2030, is a critical component in protecting both human health and the environment from being a contributor to increasing antibiotic resistance. The disparity in wastewater treatment infrastructure between urban centers and rural areas in Ecuador and within other LMICs is a significant challenge in addressing the environment as a reservoir for antibiotic-resistant bacteria and ARGs (Donoso & Rios-Touma, 2020). While urban centers can potentially benefit from higher wastewater treatment rates and funding, small rural towns, where much of the country's produce is raised, often lack access to adequate domestic wastewater treatment facilities (Egas & Ordoñez, 2010). The lack of wastewater treatment infrastructure in rural areas mixed with untreated wastewater creates important reservoirs for antibiotic-resistant bacteria and antimicrobial resistance genes, which can then spread to humans, domestic animals, and the broader environment through various exposure pathways (Gekenidis et al., 2018). The challenge to provide both piped water and domestic wastewater treatment in LMICs will become increasingly important.

There were limitations to this study. First, we were unable to assess seasonality in our sampling plan due to time and resource constraints. Second, the sample size was relatively small and the study could have detected more significant differences if larger sample sizes were used. Third, there were

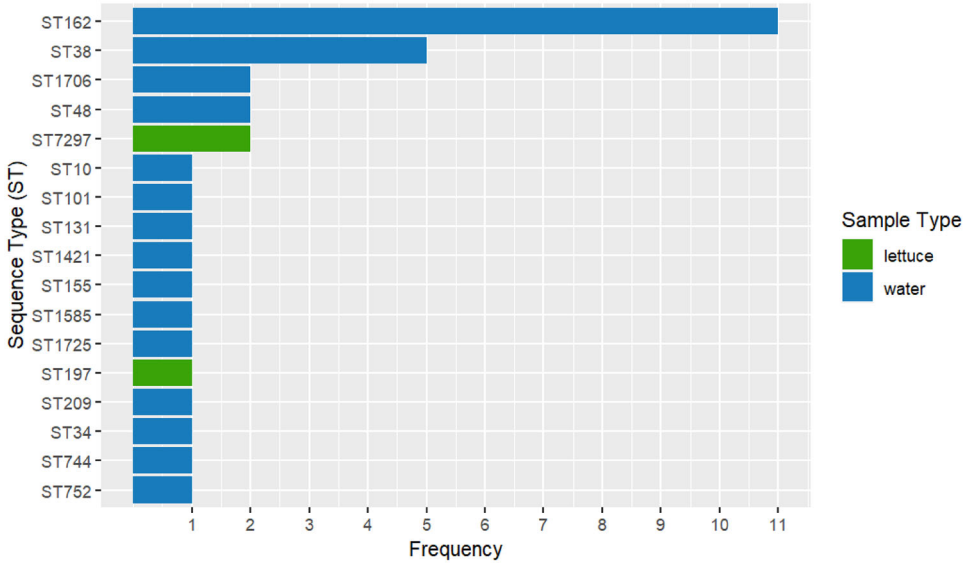

FIGURE 5 Frequency of *Escherichia coli* sequence types identified in water and lettuce *E. coli* isolates ($n = 34$).

very limited number of disperse livestock present in the watershed studied. Animal agriculture could have been a source of antibiotic-resistant bacteria and genes in the irrigation water and may have changed the dynamics of ABR transmission that cannot be attributed to human wastewater alone.

Our analysis revealed some novel findings. Among the lettuce samples, we identified two instances of *E. coli* carrying $bla_{\text{CTX-M-8}}$ genes. There were four cases of *Enterobacter cloacae* that had the same ESBL-producing gene $bla_{\text{ACT-12}}$, and two non-Enterobacterales species harboring genes associated with carbapenem resistance (bla_{OXA}-types). These observations underscore the complexity of antibiotic resistance dissemination in rural settings and the connectedness of ABR across orders and taxa.

Although our study provides valuable insights into the role of untreated wastewater in ABR dissemination, future research should consider the broader context of agricultural activities and livestock antibiotic use to develop comprehensive strategies for mitigating ABR in agricultural settings with a lack of small town wastewater treatment.

5 | CONCLUSION

The findings of our research highlight the urgent need for comprehensive development initiatives to increase domestic wastewater treatment. There were vast changes in water quality between upstream and downstream locations in the stream studied, which highlights the detrimental impact of untreated wastewater entering the environment. The significant differences in *E. coli* CFU counts between upstream and downstream samples underscored the need for improved domestic wastewater management practices. Achieving universal wastewater treatment, as outlined in the United Nations Sustainable Development Goal 6.3, is crucial for mitigating antibiotic resistance and safeguarding human health and the environment. As countries transition into middle- or upper-middle-income status, addressing this challenge becomes increasingly imperative. Effective interventions must prioritize the development of domestic wastewater treatment to prevent the spread of antibiotic resistance through irrigation water and produce and protect human health.

AUTHOR CONTRIBUTIONS

Thomas VanderYacht: Conceptualization; data curation; formal analysis; investigation; visualization; writing—original draft; writing—review and editing. **Liseth Salinas**: Formal analysis; investigation; methodology; supervision; validation; writing—review and editing. **Tilden Margaret Remerleitch**: Formal analysis; investigation; methodology. **Nikolina Walas**: Formal analysis; investigation; supervision; validation; visualization; writing—review and editing. **Tin Ho**: Formal analysis; software; supervision; visualization; writing—review and editing. **Gabriel Trueba**: Conceptualization; data curation; investigation; methodology; project administration; supervision; writing—review and editing. **Jay P. Graham**: Conceptualization; funding acquisition; investigation; methodology; resources; supervision; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

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