

Article

Antimicrobial Resistance Profiles of *Escherichia coli* Isolates from Water Samples of Hydroelectric Reservoirs in the Paraná River Basin, Brazil

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Abstract

The spread of antimicrobial-resistant bacteria in aquatic environments is a public health concern, especially in areas with anthropogenic activity. Hydroelectric reservoirs may act as interfaces facilitating the dissemination of resistance. *Escherichia coli* is used as an indicator of environmental contamination and antimicrobial resistance. To investigate the occurrence of *E. coli* in reservoirs of the Urubupungá Hydroelectric Complex (Paraná River basin, Brazil) and to characterize antimicrobial resistance profiles and ESBL/AmpC-related genes. Methods: Water samples were collected during rainy and dry seasons (2022–2024). A total of 200 *E. coli* isolates were analyzed by broth microdilution (EUCAST). ESBL and AmpC production were phenotypically assessed, and resistance genes (*bla*CTX-M, *bla*TEM, *bla*SHV, and *amp*C) were detected by PCR. 26% of isolates showed resistance to at least one antimicrobial, mainly tetracycline (18%), amoxicillin (15%), and ampicillin (15%). All isolates were susceptible to imipenem, meropenem, and norfloxacin. Among the investigated resistance markers, *amp*C was the most frequently detected gene, whereas *bla*CTX-M was the predominant ESBL-associated determinant. Significant associations were observed between *bla*CTX-M and resistance to cefotaxime, ceftazidime, and cefepime, whereas the presence of *amp*C was significantly associated with cefoxitin resistance. Significant associations were observed between β -lactam resistance and ESBL genes. Hydroelectric reservoirs under anthropogenic influence may serve as environmental reservoirs of antimicrobial-resistant *E. coli*, particularly ESBL producers. These findings highlight the role of aquatic environments in the dissemination of clinically relevant resistance genes.



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1. Introduction

Antimicrobial resistance (AMR) has emerged as a major global public health concern, driven by the widespread use of antibiotics in human and veterinary medicine [1,2]. In

addition to clinical settings, antimicrobial compounds and resistant bacteria are increasingly detected in environmental matrices, including rivers, soils, and wastewater, where they may exert selective pressure on microbial communities [3,4].

Aquatic environments receiving urban and agricultural effluents play a critical role in the dissemination of antimicrobial resistance [4,5]. In this context, *Escherichia coli* is widely used as an indicator of fecal contamination and anthropogenic impact and is frequently associated with multidrug resistance and the presence of mobile genetic elements carrying resistance genes [6,7]. Among these, extended-spectrum beta-lactamase (ESBL)-producing *E. coli* represents a particular concern due to its ability to inactivate broad-spectrum cephalosporins [8,9].

Water reservoirs, especially those associated with hydroelectric systems, may act as environmental interfaces connecting urban wastewater, agricultural activities, and natural ecosystems, thereby facilitating the persistence and spread of antimicrobial resistance genes [10]. In Brazil, hydroelectric power generation is concentrated in the Paraná River basin, a region characterized by intense agricultural and industrial activity.

The Urubupungá Hydroelectric Complex, located in this region, receives effluents from multiple municipalities and is subject to significant anthropogenic pressure. Despite its environmental and economic relevance, data on the occurrence of antimicrobial-resistant *E. coli* in these reservoirs remain limited.

Furthermore, aquatic ecosystems are increasingly recognized within the One Health framework as important environmental reservoirs involved in the maintenance and dissemination of antimicrobial resistance determinants among human, animal, and environmental compartments [11].

Therefore, this study aimed to evaluate the presence of *Escherichia coli* in water samples from reservoirs of the Urubupungá Hydroelectric Complex, Brazil, to characterize their antimicrobial susceptibility profiles and to investigate the presence of genetic markers associated with ESBL production. To our knowledge, few studies have simultaneously evaluated phenotypic antimicrobial resistance profiles and ESBL/AmpC-associated genetic markers in *E. coli* isolated from hydroelectric reservoirs in the Paraná River Basin, contributing to the understanding of environmental antimicrobial resistance in this important aquatic system.

2. Materials and Methods

2.1. Sampling Sites

Water samples were collected in a region characterized by an Aw climate according to the Köppen–Geiger classification, corresponding to a tropical climate with well-defined rainy and dry seasons. Sampling was performed at five different time points during the rainy season (October–March) and at five different time points during the dry season (June–September) between 2022 and 2024, following the methodology described by Abraham et al. [12].

All sampling sites were located within areas influenced by the Urubupungá Hydroelectric Complex in the upper Paraná River Basin, Brazil. Samples were collected from surface reservoir waters and riverine environments associated with the hydroelectric system, rather than from sediments or surrounding terrestrial environments (Table 1 and Figure 1).

The geographic coordinates of the sampling sites are presented in Figure 1.

Sampling site 1 was located at the mouth of the Sucuriú River, an important tributary of the Paraná River. Sampling sites 2, 3, and 4 were in areas directly associated with the Paraná River and the reservoirs of the Urubupungá Hydroelectric Complex, including recreational and anthropogenically influenced environments.

Table 1. Sampling sites included in the study.

Municipality-State	Location	Latitude	Longitude	Altitude
Três Lagoas-MS ¹	Sucuriú River	20°39'07.78" S	51°47'38.30" W	278 m
Selviria-MS ²	Paraná River Recreational Beach	20°20'43.86" S	51°23'7.38" W	230 m
Ilha Solteira-SP ³	Former River Port Structure	20°24'29.75" S	51°22'19.26" W	282 m
Ilha Solteira-SP ⁴	Ilha Solteira Recreational Beach	20°23'08.29" S	51°20'40.34" W	330 m

¹ Sampling site 1; ² Sampling site 2; ³ Sampling site 3; ⁴ Sampling site 4.

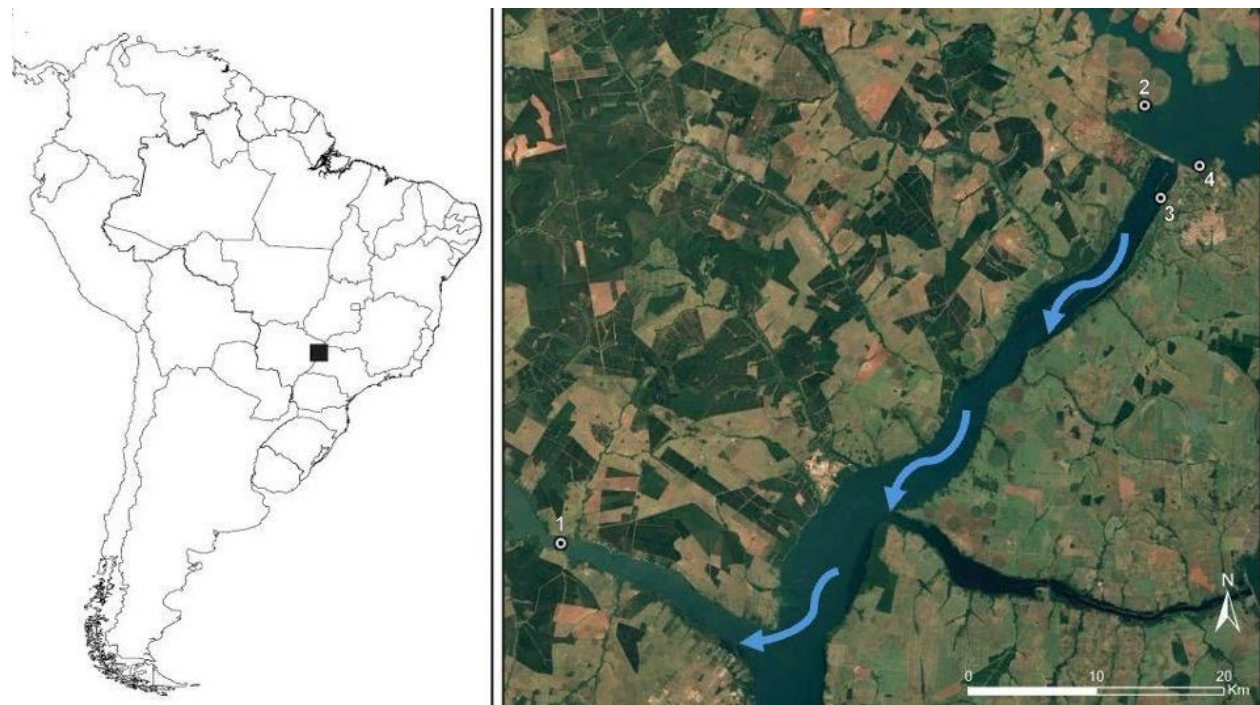


Figure 1. Sampling sites included in the study. ¹ Sucuriú River. ² Paraná River fluvial beach. ³ Structure of the former river port. ⁴ Ilha Solteira fluvial beach. The arrows indicate the direction of the river’s flow.

At each sampling site, five water samples of 1 L were collected at a depth of approximately 30 cm and at least 1 m above the bottom sediments. Samples were collected in sterile containers and transported under refrigeration (0–4 °C) until processing. All samples were processed within four hours after collection to preserve microbial viability [13,14].

2.2. Microbial Cultivation and Identification

Water samples were initially inoculated into peptone water and incubated at temperatures of 25 °C and 37 °C for three days to promote bacterial enrichment. After incubation, 0.1 mL aliquots from enriched cultures were streaked onto Eosin Methylene Blue agar and MacConkey agar (Difco Laboratories) and incubated aerobically at 37 °C for 24–48 h [15]. The use of both culture media was intended to improve the selective recovery and presumptive identification of enteric Gram-negative bacteria, particularly *Escherichia coli*.

Presumptive *Escherichia coli* colonies were selected based on characteristic lactose-fermenting morphology, including metallic green sheen colonies on Eosin Methylene Blue agar and pink to reddish colonies on MacConkey agar. Presumptive *Escherichia coli* colonies were identified based on biochemical characteristics, including citrate utilization; fermentation of lactose, glucose, and sucrose; oxidase activity; urease activity; L-lysine decarboxylase activity; motility; tryptophan deamination; gas production from glucose;

indole production; and hydrogen sulfide production. These procedures followed previously described methodologies [16,17].

Conventional biochemical identification was considered appropriate for the objectives of this environmental surveillance study and has been widely employed for the characterization of *Escherichia coli* isolated from water samples. The reference strain *Escherichia coli* ATCC 25922 was used as the quality control strain during identification procedures.

To preserve isolates throughout the sampling period, all biochemically identified *Escherichia coli* isolates were stored in Brain Heart Infusion broth (BHI) supplemented with 25% glycerol and maintained at -80°C until further analyses [18].

2.3. Antimicrobial Susceptibility Testing

Antimicrobials and Breakpoints

For antimicrobial susceptibility testing, a total of 200 *E. coli* isolates recovered during the different sampling campaigns conducted between 2022 and 2024 were included in the study. Multiple isolates were obtained throughout the sampling period from the different water samples analyzed. After microbial cultivation and biochemical identification, representative colonies presenting characteristic *Escherichia coli* morphology were selected for further analyses. Isolates recovered from independent colonies and different sampling events were considered independent isolates for antimicrobial susceptibility testing. When more than one isolate from the same taxon was available from a single water sample, a maximum of three isolates were selected for testing to reduce the possibility of repeatedly analyzing identical microorganisms.

The antimicrobial agents included in this study were selected based on their clinical relevance in human and veterinary medicine and their importance in environmental antimicrobial resistance surveillance [1,2]. The isolates were evaluated against the following antimicrobial agents: amikacin, ampicillin, amoxicillin, amoxicillin/clavulanic acid, ampicillin/sulbactam, azithromycin, aztreonam, cefadroxil, cefepime, cefotaxime, ceftazidime, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, imipenem, meropenem, norfloxacin, piperacillin, piperacillin/tazobactam, and tetracycline (Table 2).

Table 2. Clinical breakpoints for antimicrobial agents according to EUCAST.

Antimicrobial Agent	Breakpoint ($\mu\text{g/mL}$)
Amikacin	8
Amoxicillin	8
Amoxicillin/clavulanic acid ¹	8
Ampicillin	8
Ampicillin/sulbactam ²	8
Azithromycin	16
Aztreonam	4
Cefadroxil	16
Cefepime	4
Cefotaxime	2
Cefoxitin	8
Ceftazidime	4
Ceftriaxone	2
Chloramphenicol	16
Ciprofloxacin	0.5
Gentamicin	2
Imipenem	4
Meropenem	8
Norfloxacin	0.5
Piperacillin	8
Piperacillin/tazobactam ³	8
Tetracycline	4

¹ Amoxicillin/clavulanic acid: for susceptibility testing purposes, the clavulanic acid concentration is kept fixed at $2\text{ }\mu\text{g/mL}$. ² Ampicillin/sulbactam: for susceptibility testing purposes, the sulbactam concentration is kept fixed at $4\text{ }\mu\text{g/mL}$. ³ Piperacillin/tazobactam: for susceptibility testing purposes, the tazobactam concentration is kept fixed at $4\text{ }\mu\text{g/mL}$.

This panel included representatives of the main antimicrobial classes used for the treatment of infections caused by Gram-negative bacteria and enabled the investigation of resistance patterns relevant to both public health and environmental monitoring.

Clinical breakpoints established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were adopted for interpretation of antimicrobial susceptibility results [19].

2.4. Determination of Minimum Inhibitory Concentrations (MIC)

Minimum inhibitory concentrations (MICs) were determined using the broth microdilution method in sterile 96-well microtiter plates containing Mueller–Hinton broth. Each bacterial isolate was initially cultured in 2 mL of Mueller–Hinton broth and incubated at 37 °C for 12 h. The turbidity of each culture was adjusted to the 0.5 McFarland standard, corresponding to approximately $1\text{--}2 \times 10^8$ CFU/mL. The bacterial suspension was subsequently diluted to obtain a final inoculum of approximately 5×10^5 CFU/mL per well according to EUCAST recommendations [19]. Antimicrobial agents were tested using twofold serial dilutions ranging from 0.03 µg/mL to 512 µg/mL.

Visible bacterial growth was determined by visual inspection of turbidity in each well after incubation and comparison with positive growth controls and negative sterility controls. The MIC was defined as the lowest concentration of antimicrobial agent capable of inhibiting visible bacterial growth after incubation.

Microplates were incubated aerobically at 37 °C for 24 h, and isolates were classified as susceptible or resistant according to EUCAST clinical breakpoints [19]. The strain *Escherichia coli* ATCC 25922 was used as a quality control strain to ensure reliability and reproducibility of antimicrobial susceptibility testing. All experiments were performed in duplicate. MIC₅₀ and MIC₉₀ values were calculated as the minimum antimicrobial concentrations required to inhibit 50% and 90% of the isolates, respectively, based on the distribution of MIC values obtained for the tested population.

2.5. Detection of Possible Extended-Spectrum β -Lactamases (ESBL) and AmpC

Phenotypic confirmation of ESBL production was performed according to the Clinical and Laboratory Standards Institute (2010) [20] recommendations using the combination disk method. Isolates showing an increase of ≥ 5 mm in the inhibition zone diameter for ceftazidime or cefotaxime tested in combination with clavulanic acid, compared with the antimicrobial alone, were interpreted as ESBL producers.

Screening for possible ESBL production was performed based on microbial growth at or above screening concentrations ($\text{MIC} \geq 2$ µg/mL) for aztreonam, ceftazidime, cefotaxime, or ceftriaxone [21]. Phenotypic confirmation of ESBL production was performed using disk diffusion assays with cefotaxime (30 µg) and cefotaxime/clavulanic acid (30 µg/10 µg) disks (Cefar Diagnostics, São Paulo, SP, Brazil). Disks were placed equidistantly on Mueller–Hinton agar plates inoculated with bacterial suspensions. After incubation at 37 °C for 16–24 h, inhibition zones were measured.

Clavulanic acid acts as a beta-lactamase inhibitor and enhances the activity of cefotaxime against ESBL-producing isolates. ESBL production was considered positive when the inhibition zone diameter of the cephalosporin/clavulanic acid combination was at least 5 mm larger than that observed for cefotaxime alone [22].

To further confirm ESBL production, a broth microdilution assay was performed using cefotaxime (0.25–64 µg/mL) and cefotaxime combined with clavulanate (0.25/4–64/4 µg/mL). Phenotypic confirmation was defined as a reduction of at least three twofold serial dilutions in the MIC of the cephalosporin in the presence of clavulanic acid. Screening for AmpC β -lactamase production was performed using ceftioxin (30 µg) disks. Inhibition

zone diameters ≤ 19 mm were considered indicative of potential *AmpC*-producing isolates. The reference strains *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as negative and positive ESBL controls, respectively.

2.6. Detection of Genetic Markers Associated with β -Lactamases (ESBL and *AmpC*)

Isolates suspected of producing ESBL or *AmpC* β -lactamases were subjected to genomic DNA extraction for molecular detection of resistance genes. Total bacterial DNA was extracted using the Mericon DNA Bacteria Kit (QIAGEN, São Paulo, SP, Brazil), and DNA samples were stored at -80°C in ultrapure water until analysis. The presence of the genes *ampC*, *blaTEM*, *blaCTX-M*, and *blaSHV* was investigated using polymerase chain reaction (PCR) using previously described primer sets and amplification conditions reported by Rezaei et al. [23], Maheshwari et al. [24], and Fouhy et al. [25].

These genes were selected because they represent the principal beta-lactamase determinants commonly associated with resistance to penicillins, cephalosporins, and extended-spectrum beta-lactam antibiotics in *Enterobacterales*.

PCR amplification was performed using an AmpliTherm Thermal Cycler (EPICENTRE Biotechnologies, Madison, WI, USA) with an initial denaturation step at 94°C for 5 min, followed by 35 amplification cycles consisting of denaturation at 94°C for 1 min, primer-specific annealing temperature for 1 min, and extension at 72°C for 1 min. A final extension step at 72°C for 5 min was included. For the phenotypic β -lactamase assays, *Staphylococcus aureus* ATCC 29213 and *Klebsiella pneumoniae* ATCC 700603 were used as positive controls. *K. pneumoniae* ATCC 700603 was also used as the positive control for the phenotypic ESBL assay.

Ultrapure water was used as the negative control in all PCR reactions. PCR products were analyzed by electrophoresis on 1% agarose gels stained with ethidium bromide ($0.5\text{ }\mu\text{g/mL}$) and visualized under UV transillumination (Kodak EDA-System 120). A 1 kb DNA ladder (Gibco, São Paulo, SP, Brazil) was used as a molecular size marker (Table 3).

Table 3. Primer pairs used for amplification of genes associated with β -lactamase production in *Escherichia coli*.

Target Gene	Primer Sequence (5'–3')	Annealing Temperature ($^{\circ}\text{C}$)	Amplicon Size (bp)	Reference
<i>ampC</i>	F: CCCCCTTATAGAGCAACAA R: TCAATGGTCGACTTCACACC	50	593	Rezaei et al. [22]
<i>blaCTX-M</i>	F: TTTGCGATGTGCAGTACCAGTAA R: CGATATCGTTGGTGGTGCCATA	51	544	Maheshwari et al. [23]
<i>blaSHV</i>	F: ATTTGTCGCTTCTTACTCGC R: TTTATGGCGTTACCTTGACC	55	1051	Fouhy et al. [24]
<i>blaTEM</i>	F: ATGAGTATTCAACATTTCCGTG R: TTACCAATGCTTAATCAGTGAG	55	840	Fouhy et al. [24]

Presumptive colonies were identified using selective culture media and biochemical-physiological phenotypic tests [12]. The isolates subjected to molecular detection of β -lactam resistance genes were the same isolates previously selected for the phenotypic evaluation of β -lactamase production and for extended-spectrum β -lactamase (ESBL) screening. Thus, the molecular investigation was specifically directed toward isolates exhibiting phenotypic resistance to β -lactam antimicrobials or results suggestive of β -lactamase production, allowing direct comparison between phenotypic and genotypic resistance profiles.

To ensure the reliability of the phenotypic assays, ATCC 700603 was used as the positive control strain for ESBL detection, whereas ATCC 29213 and ATCC 700603 were used as positive controls in the β -lactamase production assays. All tests were performed in dupli-

cate and were considered valid only when the control strains produced the expected results. The isolates characterized phenotypically were subsequently evaluated for the presence of the *ampC*, *blaCTX-M*, *blaTEM*, and *blaSHV* genes, enabling comparison between phenotypic resistance mechanisms and the corresponding genetic determinants of β -lactam resistance.

Primer sequences, annealing temperatures, expected amplicon sizes, and respective references are presented in Table 3.

2.7. Statistical Analysis

Statistical analyses were performed using the Chi-square test with Monte Carlo correction for variables with low prevalence values. Associations between binary variables were evaluated using the Phi–Pearson correlation coefficient. The distribution of resistant microorganisms and resistance markers was also evaluated using the Kruskal–Wallis test. Statistical significance was established at $p < 0.05$.

3. Results

Table 4 presents the results of antimicrobial susceptibility testing. Among the isolates analyzed, 52 (26%) showed resistance to at least one antimicrobial agent. Resistance was most frequently observed among β -lactam antibiotics, particularly amoxicillin (15%), ampicillin (15%), and piperacillin (12%), as well as tetracycline (18%). Overall, resistance to β -lactam antimicrobials was frequently associated with resistance to other drugs within the same class.

Table 4. Antimicrobial susceptibility of 200 *Escherichia coli* isolates obtained from watercourses.

Antimicrobial	MIC Range ¹ (μ g/mL)	MIC ₅₀ ²	MIC ₉₀ ³	Resistance (%)
Amikacin	0.03–128.0	1.0	4.0	1.5
Amoxicillin	0.125–512.0	2.0	64.0	15.0
Amoxicillin/Clavulanic acid	0.125–256.0	1.0	8.0	8.0
Ampicillin	0.06–128.0	1.0	128.0	15.0
Ampicillin/Sulbactam	0.06–128.0	1.0	8.0	8.0
Azithromycin	0.125–64.0	1.0	4.0	9.0
Aztreonam	$\leq$ 0.03–32.0	0.125	2.0	0.5
Cefadroxil	1.0–256.0	4.0	8.0	5.5
Cefepime	$\leq$ 0.03–512.0	0.06	4.0	4.0
Cefotaxime	$\leq$ 0.03–256.0	0.06	2.0	2.5
Cefoxitin	0.125–256.0	2.0	4.0	4.5
Ceftazidime	0.06–256.0	0.125	4.0	3.0
Ceftriaxone	$\leq$ 0.03–16.0	$\leq$ 0.03	0.25	1.0
Chloramphenicol	0.5–128.0	1.0	8.0	2.5
Ciprofloxacin	$\leq$ 0.03–128.0	$\leq$ 0.03	0.06	1.5
Gentamicin	$\leq$ 0.03–128.0	$\leq$ 0.03	0.06	3.0
Imipenem	$\leq$ 0.03–1.0	$\leq$ 0.03	0.25	0.0
Meropenem	$\leq$ 0.03–0.5	$\leq$ 0.03	0.125	0.0
Norfloxacin	$\leq$ 0.03–0.5	$\leq$ 0.03	0.25	0.0
Piperacillin	0.06–512.0	1.0	32.0	12.0
Piperacillin/Tazobactam	$\leq$ 0.03–512.0	1.0	8.0	4.0
Tetracycline	0.06–512.0	0.5	32.0	18.0

¹ MIC range: Minimum inhibitory concentration range. ² MIC₅₀: minimum inhibitory concentration required to inhibit 50% of the isolates. ³ MIC₉₀: minimum inhibitory concentration required to inhibit 90% of the isolates.

Antimicrobial susceptibility was assessed by the broth microdilution method to determine the minimum inhibitory concentration (MIC) [26].

Among all antimicrobials tested, imipenem, meropenem, and norfloxacin remained active against all isolates. Among the isolates resistant to at least one β -lactam antimicrobial, resistance patterns varied considerably. Two isolates (6.5%) were resistant to eight β -lactams, twelve isolates (38.7%) were resistant to seven β -lactams, five isolates (16.1%)

were resistant to six β -lactams, nine isolates (29%) were resistant to five β -lactams, three isolates (9.7%) were resistant to four β -lactams, and one isolate (3.8%) was resistant to three β -lactams.

PCR screening was performed exclusively on the 48 isolates that showed phenotypic evidence suggestive of ESBL and/or AmpC production. Consequently, isolates without phenotypic evidence of β -lactamase production were not included in the molecular analysis. Therefore, the PCR results represent the frequency of resistance genes within the phenotypically selected subset and do not allow complete phenotype–genotype concordance analysis.

Among all antimicrobials tested, imipenem and meropenem (both carbapenems), as well as norfloxacin (a fluoroquinolone), showed complete effectiveness against all isolates. Resistance to aztreonam was detected in only one isolate (0.5%).

Most resistant isolates presented unique resistance patterns, indicating considerable diversity among the resistant *E. coli* population recovered from the studied aquatic environments.

Among the 52 resistant isolates, 32 (61.5%) exhibited unique antimicrobial resistance profiles. Repeated resistance patterns were mainly observed among isolates resistant to one or two antimicrobials (18 isolates; 34.6%), except for two isolates resistant to six antimicrobials obtained from different sampling sites and collection periods (Table 5).

Table 5. Different antimicrobial resistance profiles observed among 52 resistant isolates.

Resistance Profile	<i>n</i>
AZM	1
TET	12
AZM + TET	4
GEN + TET	1
CHL + TET	2
AZM + CHL + TET	1
AMP + AMX + AZM + PIP	1
AMP + AMX + CFR + TET	1
AMP + AMX + AZM + PIP + TZP	1
AMC + AMP + AMX + PIP + SAM	1
AMP + AMX + CAZ + CTX + TET	1
AMP + AMX + AZM + CHL + TET	1
AMP + AMX + AZM + PIP + SAM + TZP	1
AMP + AMX + AZM + PIP + TET	1
AMC + AMP + AMX + AZM + PIP + SAM	1
AMK + AMP + AMX + CFR + GEN + TET	1
AMP + AMX + CFR + CTX + FEP + PIP	2
AMP + AMX + CAZ + CFR + FEP + TET	1
AMP + AMX + CAZ + CRO + FEP + TET	1
AMC + AMP + AMX + AZM + PIP + SAM + TET	1
AMC + AMP + AMX + FOX + PIP + SAM + TZP	3
AMC + AMP + AMX + CIP + PIP + SAM + TET	2
AMP + AMX + CAZ + CFR + CTX + FEP + PIP + TET	1
AMC + AMP + AMX + AZM + FOX + PIP + SAM + TZP	3
AMC + AMP + AMX + CFR + FOX + GEN + PIP + SAM	1
AMC + AMP + AMX + CFR + FOX + PIP + SAM + TZP	1
AMP + AMX + CAZ + CFR + CRO + FEP + PIP + TET	1
AMP + AMX + CAZ + CFR + CTX + FEP + PIP + TET	2
AMP + AMX + ATM + AZM + CAZ + CFR + CIP + CTX + GEN	1
AMC + AMK + AMP + AMX + FOX + GEN + PIP + SAM + TET	1

Abbreviations: AMK, amikacin; AMC, amoxicillin/clavulanate; AMP, ampicillin; AMX, amoxicillin; ATM, aztreonam; AZM, azithromycin; CAZ, ceftazidime; CFR, cefadroxil; CHL, chloramphenicol; CIP, ciprofloxacin; CRO, ceftriaxone; CTX, cefotaxime; FEP, cefepime; FOX, ceftiofur; GEN, gentamicin; PIP, piperacillin; SAM, ampicillin/sulbactam; TET, tetracycline; TZP, piperacillin/tazobactam.

No statistically significant differences were observed in the distribution of resistant isolates among the sampling sites or between collection periods ($p \geq 0.05$). Therefore, the results were analyzed without stratification by sampling location.

Correlation analyses revealed strong associations between resistance to amoxicillin/clavulanic acid and ampicillin/sulbactam ($\phi = 1.0$), as well as between resistance to amoxicillin and ampicillin ($\phi = 0.961$). Additional correlations were observed between ampicillin and piperacillin resistance ($\phi = 0.793$) and between amoxicillin and piperacillin resistance ($\phi = 0.762$) (Figure 2).

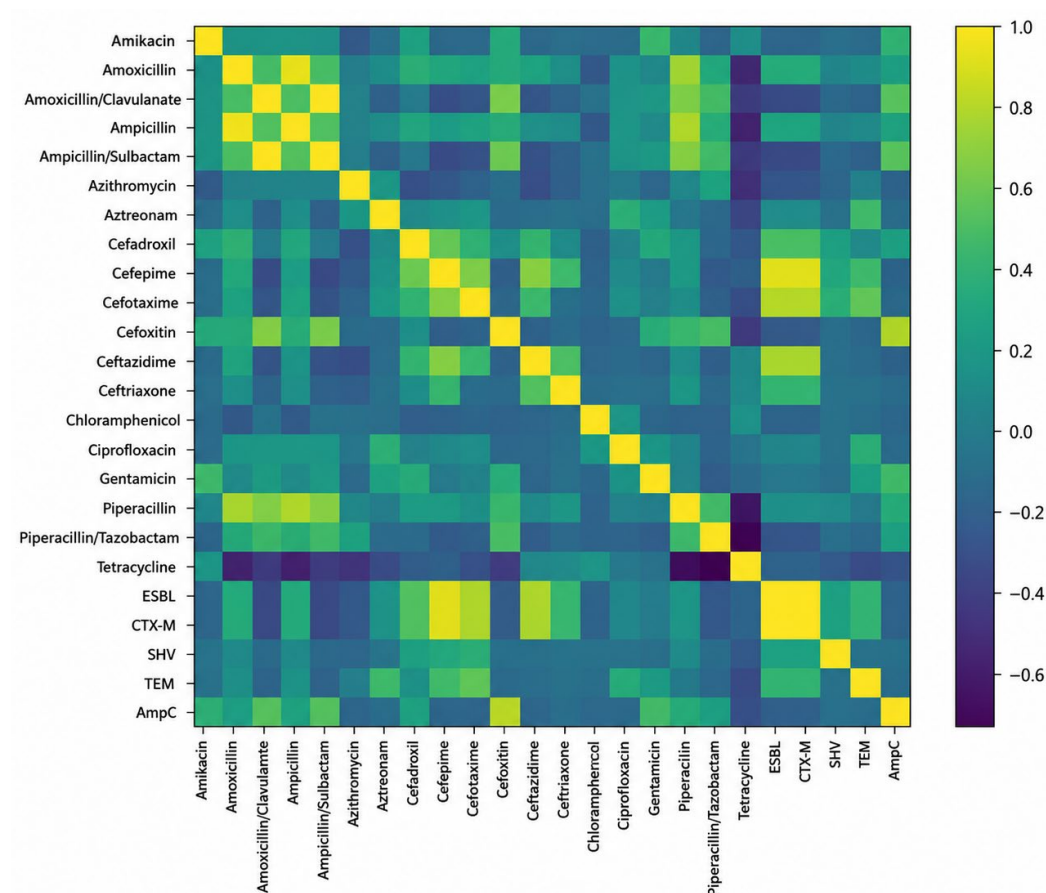


Figure 2. Correlation matrix among the studied variables: antimicrobial resistance and detected resistance markers.

The occurrence of beta-lactamase-associated genes among *E. coli* isolates evaluated for beta-lactamase markers is summarized in Table 6. Among these isolates ($n = 48$), *ampC* was detected in 36 isolates (75.0%), *blaCTX-M* in 8 isolates (16.7%), *blaTEM* in 5 isolates (10.4%), and *blaSHV* in 2 isolates (4.2%). Thus, *ampC* was the most frequent marker detected, whereas *blaCTX-M* was the most frequent ESBL-associated gene.

Table 6. Distribution of beta-lactamase-associated genes among *E. coli* isolates evaluated by PCR.

Genetic Marker	Positive Isolates n (%)	Main Interpretation	Reference
<i>ampC</i>	36/48 (75.0%)	Most frequent beta-lactamase-associated marker	Rezaei et al. [22]
<i>blaCTX-M</i>	8/48 (16.7%)	Most frequent ESBL-associated marker	Maheshwari et al. [23]
<i>blaTEM</i>	5/48 (10.4%)	Detected less frequently	Fouhy et al. [24]
<i>blaSHV</i>	2/48 (4.2%)	Least frequent among investigated markers	Fouhy et al. [24]

Percentages were calculated among the 48 *E. coli* isolates submitted to molecular screening for beta-lactamase-associated markers. PCR screening was performed only on the 48 isolates previously selected based on phenotypic evidence suggestive of ESBL or AmpC production. Therefore, all gene frequencies reported refer exclusively to this PCR-screened subset and should not be interpreted as prevalence among the entire collection of 200 *E. coli* isolates. The 200 *E. coli* isolates analyzed in this study were obtained from water samples collected at four sampling sites during both dry and rainy seasons. The isolates originated from all sampling sites and collection periods.

Because PCR analysis was restricted to the 48 isolates selected based on phenotypic evidence suggestive of ESBL or AmpC production, the detected gene frequencies should be interpreted only within this phenotypically selected subset. Phenotypically negative isolates were not evaluated by PCR. The 48 isolates included in the molecular analysis were selected exclusively according to their phenotypic evidence of ESBL and/or AmpC production, irrespective of sampling site or collection period. Because the original database did not retain the distribution of these selected isolates according to sampling site and season, these data cannot be provided retrospectively.

Up to three representative colonies were selected from each water sample for antimicrobial susceptibility testing to reduce redundant testing of isolates with similar phenotypic characteristics. However, because molecular typing was not performed, isolates recovered from the same water sample cannot be assumed to be clonally independent.

No statistically significant associations were observed between resistance to penicillins and the presence of ESBL or *ampC* genes. Among β -lactamase-related markers, the presence of *bla*CTX-M was representative of all ESBL markers investigated ($\phi = 1.0$). A strong correlation was observed between *bla*CTX-M and resistance to cefepime ($\phi = 0.932$), as well as resistance to cefotaxime or ceftazidime ($\phi = 0.789$). A correlation was also observed between ceftazidime resistance and the presence of *ampC* markers ($\phi = 0.847$).

4. Discussion

Antibiotic resistance is considered one of the most significant threats to global public health, and bacteria can employ a wide range of mechanisms to evade the action of antimicrobial agents present in the environment [1,27]. These mechanisms include non-specific processes such as increased activity of efflux pumps and reduced permeability of the outer membrane due to downregulation of porins, as well as specific mechanisms such as the production of enzymes capable of inactivating antibiotics [28,29].

Such phenomena are frequently associated with the acquisition and dissemination of resistance genes through mobile genetic elements and contribute to the development and spread of antimicrobial resistance in clinical and hospital environments [1,30].

The distribution of resistant microorganisms in water samples from reservoirs of the Urubupungá Hydroelectric Complex did not show significant influence from sampling period or location. This result suggests that contamination by resistant *Escherichia coli* occurs relatively constantly in these environments, indicating that contamination sources are likely continuous rather than sporadic. This finding may reflect the persistent influence of anthropogenic activities in the study area, including agricultural production, livestock farming, recreational water use, and urban effluents [5,31,32].

Although no statistically significant differences were observed in the distribution of antimicrobial-resistant microorganisms among the sampling sites or between sampling periods, the findings should be interpreted considering the distinct environmental characteristics of the study areas. The sampling site located at the mouth of the Sucuriú River represents an important tributary of the Paraná River, whereas the remaining sites are directly associated with the Paraná River and the reservoirs of the Urubupungá Hydroelectric

Complex, areas characterized by intensive recreational use, economic activities, and public water supply.

These environments are embedded within a landscape under substantial anthropogenic influence, characterized by agricultural expansion, livestock production, urban development, and recreational activities. Such conditions may promote the continuous introduction of antimicrobial-resistant bacteria and resistance genes through multiple pathways, including domestic wastewater discharge, agricultural runoff, drainage from rural properties, and tributaries flowing through populated areas. Therefore, even in the absence of statistically significant differences among the sampling sites, the widespread anthropogenic pressure throughout the watershed likely contributes to the relatively homogeneous distribution of the resistance determinants observed.

Although the Sucuriú River is less urbanized than the areas directly associated with the Paraná River and its reservoirs, it is also influenced by extensive agricultural activities within its drainage basin. Surface runoff may transport nutrients, microorganisms, and contaminants, facilitating the dissemination of resistant bacteria downstream toward its confluence with the Paraná River. In contrast, the reservoir sites are simultaneously exposed to multiple potential contamination sources, including greater human activity, boat traffic, recreational water use, and inputs from urban wastewater, all of which have been associated with increased loads of antimicrobial-resistant microorganisms in aquatic ecosystems.

The prevalence of resistant isolates (26%) was considerable, although lower than that reported in previous studies conducted in aquatic environments. This finding suggests that although selective pressure exerted by antimicrobials is evident in water bodies receiving urban and agricultural effluents, resistance prevalence in water reservoirs may vary depending on local factors such as contamination load, dilution processes, and microbial population dynamics.

Among the antimicrobials evaluated, tetracycline showed the highest resistance rate (18%), followed by amoxicillin and ampicillin (15% each). This pattern has been widely described in the literature and may be related to the extensive horizontal dissemination of tetracycline resistance genes among different bacterial species. Similar findings have been reported in environmental studies evaluating *Escherichia coli* from aquatic ecosystems, where tetracycline and beta-lactam resistance are frequently observed [31–33].

These antimicrobial classes are widely used both in clinical practice and in animal production systems, particularly in intensive livestock farming, where tetracyclines and β -lactams have historically been used as therapeutic agents and, in some cases, as growth promoters.

The diversity of resistance profiles observed suggests multiple origins for resistant isolates and distinct antimicrobial selection events. The absence of differences among sampling sites suggests that the reservoir functions as an integrated ecological system in which water circulation promotes homogenization of microbial populations.

The detection of beta-lactamase-associated genes, particularly *ampC* and *blaCTX-M*, reinforces the role of aquatic environments as reservoirs of clinically relevant antimicrobial resistance determinants. Although *ampC* was the most frequently detected marker, *blaCTX-M* was the main ESBL-associated gene identified among the isolates. *blaTEM* and *blaSHV* were also detected, but at lower frequencies. CTX-M-type enzymes have become the most prevalent ESBL family worldwide and are frequently detected in environmental isolates originating from aquatic ecosystems. No resistance was detected to carbapenems, which is consistent with previous studies indicating that carbapenem resistance in environmental *E. coli* isolates remains relatively rare. The occurrence of these genes in environmental isolates highlights the importance of monitoring aquatic ecosystems as potential reservoirs and dissemination routes of antimicrobial resistance determinants [33–35].

In addition to point-source contamination, reservoirs exhibit hydrodynamic characteristics distinct from those of free-flowing rivers. Longer water residence times and reduced flow velocity may favor the persistence of environmental bacteria and the maintenance of antimicrobial resistance genes within microbial communities, thereby increasing opportunities for horizontal gene transfer. Consequently, although no statistical differences were detected among the sampling sites, the specific environmental characteristics of each location are likely to influence ecological processes involved in the persistence and dissemination of antimicrobial resistance.

These findings further indicate that the absence of statistically significant differences among the study sites should not be interpreted as evidence of the absence of environmental influence. Rather, it may reflect the high hydrological connectivity between the tributaries and the Paraná River, combined with the widespread anthropogenic occupation of the watershed. Such conditions may facilitate the continuous dispersal of resistant microorganisms among aquatic environments, reinforcing the role of surface waters as important environmental reservoirs of antimicrobial resistance genes. Accordingly, our findings support the growing body of evidence indicating that human-impacted aquatic ecosystems constitute important reservoirs and dissemination pathways for antimicrobial resistance, highlighting the need for continuous environmental surveillance within the One Health framework.

This study has some limitations that should be acknowledged. First, bacterial identification was based on conventional biochemical methods without molecular confirmation. Although this approach is consistent with standardized microbiological protocols, molecular confirmation could further improve taxonomic accuracy. Second, the analysis focused on phenotypic resistance and a restricted panel of β -lactamase-related genes, which may not capture the full diversity of resistance mechanisms. Third, physicochemical characteristics of the water and quantitative bacterial loads were not evaluated, limiting the assessment of environmental drivers of resistance. Finally, genomic approaches capable of assessing clonality, plasmid content, and the broader resistome were not performed. Despite these limitations, the present study provides relevant evidence that hydroelectric reservoirs may serve as environmental reservoirs of antimicrobial-resistant *E. coli* and associated resistance genes.

From a One Health perspective, these findings support the inclusion of aquatic environments in antimicrobial resistance surveillance strategies, considering the close interaction among environmental, animal, and human health compartments [11].

5. Conclusions

The phenotypic and genotypic characterization performed in this study provides relevant information regarding the antimicrobial resistance profile of *Escherichia coli* in an important Brazilian aquatic system. The occurrence of resistant isolates, particularly those carrying beta-lactamase-associated markers, reinforces the role of aquatic environments as potential reservoirs of antimicrobial resistance determinants.

These findings may contribute to the development of public health policies aimed at monitoring antimicrobial resistance in aquatic environments and to a better understanding of the mechanisms involved in the dissemination of resistance genes in natural ecosystems. In addition, the results support the inclusion of environmental waters in antimicrobial resistance surveillance strategies, consistent with the One Health perspective.

Future studies should include investigations of additional resistance determinants, such as genes associated with quinolone and aminoglycoside resistance, as well as experimental analyses of horizontal gene transfer to better understand the dissemination potential of these mechanisms in aquatic environments.

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