Asian Journal of Water, Environment and Pollution, Vol. 21, No. 6 (2024), pp. 15-22.
DOI 10.3233/AJW240068

The Antibiotic Resistance Profile of *Escherichia coli* from Selected Estuaries in the City of Manila, Philippines

Aaron Jan Palmares*, Jayme Jyles Rongalirios, Marticia Santos, Marc Joseph Siggaoat, Pauline Lourdes Sucgang, Nicole Niren Tipa, John Chrysler Adriane Torres, Caroline Anne Vasquez, Angela Mae Viernes and Francisco Gellecanao
Department of Medical Technology, Institute of Health Sciences and Nursing, Far Eastern University
Nicanor Reyes St., Manila, Philippines
✉ apalmares@feu.edu.ph

Received June 8, 2024; revised and accepted October 5, 2024

Abstract: Surface waters from estuaries were reported to disseminate antibiotic resistance (AR), particularly from faecal coliforms of human origin introduced via domestic wastewater and surface runoffs. Thus, this study aimed to determine the concentration and antimicrobial susceptibility profiles of *Escherichia coli* isolated from surface waters of selected estuaries in the City of Manila. From a total of 48 water samples collected from six estuaries over a two-month period, the estimated average concentration of *E*. coli and total coliform are 4.3 x 10^4 CFU/100 mL and 11.0 × 10^4 CFU/100 mL, respectively. Among the isolates, 14.6% were identified as multidrug-resistant, while 6.3% were classified as extended-spectrum β-lactamase producers. Moreover, 18.8% of the isolates had a multiple antibiotic resistance index of ≥0.2. The findings indicate that AR *E. coli* contamination likely originates from human excretions, including those from informal settlements and hospital patients treated with multiple antibiotics. This makes these estuaries unsuitable for beneficial use due to the potential risk of exposure to MDR/ESBL *E. coli* and other faecal coliforms. Thus, urgent measures including law-enforced monitoring, management, rehabilitation, and education are necessary to curb faecal pollution and limit the spread of AR in these waters.

Key words: Estuary, coliform, *Escherichia coli*, antimicrobial resistance

Introduction

Surface waters of rivers, creeks, and estuaries have been reported to disseminate antibiotic resistance (AR) from *Escherichia coli* (*E. coli*) and other coliforms of human origin through domestic effluents and surface runoffs from municipalities, hospitals (Alawi et al., 2022; Azuma et al., 2022; Larsson & Flach, 2022; Sharahi et al., 2021), and wastewater treatment plants (Henriot et al., 2023; Kumar et al., 2020; Ma et al., 2022; Puljko et al., 2022; Turolla et al., 2018). This might imply that estuaries in the City of Manila are also at risk of disseminating AR from coliforms of human origin since the easements on both sides of the estuaries were obstructed with improvised shelters of informal settlement families (ISF). With no formal septage containment systems from ISFs, the estuaries were left to receive septic waste and other domestic wastewater (Ancheta, 2021; Bringula et al., 2015; Clemente, 2020). At present, the problem posed by AR bacteria is so troubling that surveillance networks were established to monitor AR (ARSP, 2023). However, these surveillance networks focus only on human isolates and not on water bodies such as estuaries. Suppose these estuaries were purposely used for recreation and domestic use; if the AR coliforms it contain caused an infection, this may

*Corresponding Author

limit the available therapeutic options. To date, there is a scarcity of research assessing the incidence of AR in the environment (Rousham et al., 2018), particularly in surface waters of estuaries. Therefore, this study aims to determine the concentration, antimicrobial resistance profile, and multiple antibiotic resistance (MAR) index of *E. coli* isolated in waters from highly urbanised estuaries in the City of Manila, Philippines.

Material and Methods

Sample Collection

Six estuaries were selected within the City of Manila. The city has an estimated 1.85 million residents within an area of 42 km^2 or more than 44,000 residents/km^2 (Morley, 2018). For each of the six estuaries (Estero de Binondo, Estero de Paco, Estero de Sampaloc, Estero de San Lazaro, Estero de SunogApog, and Estero de Valencia), four grab water samples (100 mL each) were collected at depths ranging from 1 to 15 cm (see Figure 1). Two rounds of sampling were conducted, the first sampling in the first week of February 2024 and the second sampling in the first week of March 2024. In total, 48 samples were collected with eight samples obtained from each estuary. All grab water samples were collected into sterile conical tubes, immediately placed on ice, and transported to the laboratory for processing within four hours of collection.

Figure 1: Map of the City of Manila showing the estuary location of (a) Estero de Sunog Apog (b) Estero de San Lazaro, (c) Estero de Sampaloc, (d) Estero de Binondo, (e) Estero de Valencia, and (f) Estero de Paco.

Microbial Quality Testing

Each water sample was subjected to a selective isolation of *E. coli* and other coliforms using the 3M Petrifilm *E. coli*/Coliform Count (EC) Plate following the manufacturer's instruction. Briefly, a 1-mL aliquot of a 5:1000 dilution (prepared with sterile 0.9% saline solution) was dispensed onto the EC Plate. The EC plate was then incubated at 37±0.1ºC for 48±1 hours with maximum stacks of four plates. After incubation, colonies were counted using a colony counter. Blue colony-forming units (CFUs) associated with gas bubbles were considered presumptive *E. coli*, while both blue and red CFUs with gas bubbles were counted as total coliforms (Azuma et al., 2022).To calculate CFUs per mL, colony counts were multiplied by the dilution factor of 200. For the final CFU count per 100 mL, the results were further multiplied by 100 (Chen et al., 2017).

Subculture of Presumptive *E. coli* Colonies

Representative blue colonies with gas bubbles that grew in EC plates were identified and further determined for their antimicrobial susceptibility profile. Briefly, four to five presumptive *E. coli* colonies in EC plates were subcultured onto Eosin Methylene Blue (EMB) agar (Hi Media Laboratories, Mumbai, India) by isolation streaking. The EMB agar was then incubated at 37±0.5ºC for 24 hours. On EMB agar, presumptive *E. coli* colonies appear purple with or without a green metallic sheen. Representative four to five well-isolated colonies with green metallic sheen were further subcultured onto MacConkey (MAC) agar (HiMedia Laboratories, Mumbai, India) by isolation streaking. The MAC agar was then incubated at 37±0.5ºC for 18 hrs. On MAC agar, presumptive colonies of *E. coli* appear as pink colonies with or without a pink halo (Palmares & De los Reyes, 2016).

Identification and Antimicrobial Susceptibility Testing

Bacterial suspensions were prepared from presumptive *E. coli* colonies on MAC agar by emulsifying the colonies in 0.5% sodium chloride. Using the VITEK DensiCHEK^{TM} (bioMérieux), the turbidity of the bacterial suspension was adjusted to match the 0.55 McFarland standard. Afterward, the bacterial suspension and the VITEK 2 ID-N261 card were placed into the VITEK 2 system (bioMérieux, Durham, NC, USA) for Identification. Antibiotic susceptibility testing (AST) was then performed by VITEK 2 using the AST-N261 card with software version 9.03.3, according to the manufacturer's instructions. The VITEK 2 ESBL (Extended-Spectrum Beta-Lactamase) test was included on the AST-N261 card for *E. coli*. Isolates were tested with sixteen antibiotics covering six different antimicrobial classes or subclasses. This includes β-lactams such as: a. penicillin's [amoxicillin/clavulanic acid (AMC), ampicillin (AMP), and piperacillin/tazobactam (TZP)], b. cephalosporins [cefepime (FEP), cefoxitin (FOX), ceftazidime (CAZ), ceftriaxone (CRO), cefuroxime (CXM), and cefuroxime axetil (CXMA)] and, c. carbapenems [(ertapenem (ETP), imipenem (IMP), and meropenem (MEM)], d. aminoglycosides such as amikacin (AMK) and gentamicin (GEN), e. quinolones such as ciprofloxacin (CIP), and f. sulfonamides such as cotrimoxazole (SXT). The isolates were then classified as resistant, intermediate, or sensitive based on their minimum inhibitory concentrations (MIC's) following the CLSI (Clinical and Laboratory Standards Institute) guidelines (CLSI, 2021).

Calculation of Multiple Antibiotic Resistance Indices

The multiple antibiotic resistance (MAR) index of *E. coli* was then calculated using the formula: MAR index = a/b, where 'a' refers to the number of antibiotics to which *E. coli* showed resistance, and 'b' refers to the total number of antibiotics to which the *E. coli* was tested (Titilawo et al., 2015). In addition, the isolates were further classified according to their AR levels, such as antibiotic-resistant (AR, if resistant to 1 or 2 antimicrobials); multiple antibiotic-resistant (MAR, if resistant to ≥3 antimicrobials), and multidrug-resistant (MDR, if resistant to at least one antimicrobial belonging to ≥3 different classes/subclasses). The isolates were also classified as ESBL producers if they exhibited resistance to at least two out of three tested cephalosporins: CAZ, CRO, CXM (ARSP, 2023; Ham et al., 2012).

Data Analysis

The association between the collection month and the isolation frequencies of ESBL, MAR, and MDR *E. coli*, as well as the significant differences in the group means of *E. coli* and coliform concentration between the month/site of collection, were analyzed using the SPSS version 28.0 (SPSS Inc., Chicago, IL, USA). Fisher's exact test was employed for associating categorical data, while T-test or ANOVA was used for comparing the group means. Tukey's post-hoc test was performed for further comparison. A p-value of ≤0.05 was considered statistically significant.

Results and Discussion

Coliform and *E. coli* Concentrations from the Water Samples

Water samples collected from the six estuaries have an estimated average *E. coli* and coliform concentrations of 4.3×10^4 CFU/100 mL and 11.0×10^4CFU/100 mL, respectively. Detailed concentrations from each site and sampling month are presented in Table 1. Moreover, post-hoc comparisons revealed a significant difference only between the coliform concentrations of Estero de Valencia and Estero de Sunog Apog (14.7×10^4 CFU/100 mL vs. 7.7×10^4 CFU/100 mL, $p = 0.027$). Regardless, all estuaries have far exceeded the upper microbial limit of 100, 200, and 400 CFU/100 mL of *E. coli* for Class B (primary contact recreation), Class C (fishery, non-contact recreation, industrial), and Class D (navigable) water, respectively (DENR, 2015, 2016; EPA, 2012; "Guidelines on Recreational Water Quality. Volume 1: Coastal and Fresh Waters. Geneva," 2021). This indicates severe faecal contamination, rendering the estuaries unsuitable for any beneficial use (Clemente, 2020). Furthermore, the seasonal analysis showed that water samples collected in March, during the hot dry season, had significantly higher *E. coli* concentrations compared to those collected in February, which falls within the cool dry season ($P \leq 0.001$), see Table 1. Previous studies have demonstrated that seasonal variations, particularly higher temperatures, enhance *E. coli* growth and survival in aquatic environments (Abia et al., 2015; Garcia et al., 2015; Sanderson et al., 2018; Stocker et al., 2019). In this study, however, the higher *E. coli* levels in March could also be linked to increased septic waste and domestic wastewater discharge into the estuaries.

The Antimicrobial Susceptibility Profile of *E. coli*

Forty-eight *E. coli* isolates were subjected to AST. The antibiotics with the highest resistance rates were AMP, SXT, CIP, and CXM, with 54.2%, 45.8%, 16.7%, and 6.3%, respectively (see Figure 2). However, all these 48 *E. coli* isolates were susceptible to AMK, FEP, and FOX and thus were excluded from Figure 2. The ranking of resistance rates aligns with the data of the 2022

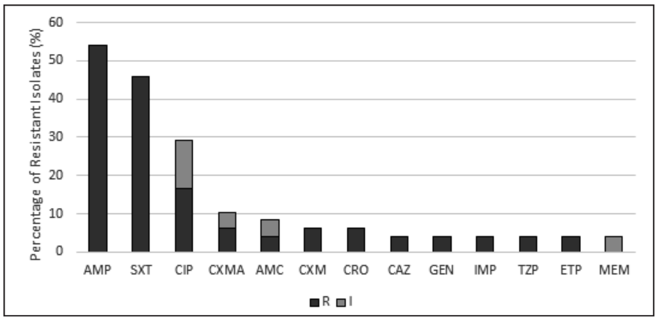

Figure 2: Antibiotic resistance profile of E. coli from water samples. AMC, amoxicillin/clavulanic acid; AMP, ampicillin; SXT, sulfamethoxazole-trimethoprim; CIP, ciprofloxacin; CXMA, cefuroxime axetil; CXM, cefuroxime; CRO, ceftriaxone; CAZ, ceftazidime; GEN, gentamicin; IMP, imipenem; TZP, piperacillin/tazobactam; ETP, ertapenem; MEM, meropenem; R, resistant; I, Intermediate.

Table 1: Concentration of E. coli and coliforms according to site and month of collection

Variables	*Categories*	*E. coli CFU/100 mL Mean ± SD (10^4)*	*Coliform CFU/100 mL Mean ± SD (10^4)*
Site	Estero de Binondo	4.7 ± 2.5	12.2 ± 4.8
	Estero de Paco	5.1 ± 2.4	14.1 ± 6.7
	Estero de Sampaloc	3.7 ± 1.1	8.5 ± 3.3
	Estero de San Lazaro	4.8 ± 2.0	8.7 ± 3.5
	Estero de Sunog Apog	2.6 ± 1.2	7.7 ± 2.1
	Estero de Valencia	4.7 ± 2.2	14.7 ± 4.9
	p-value	0.133	0.005
Month (Season)	February (Cool Dry)	3.3 ± 1.9	11.5 ± 6.4
	March (Hot Dry)	5.3 ± 1.7	10.5 ± 3.0
	p-value	≤0.001	0.482

CFU, colony forming units; SD, standard deviation

Antimicrobial Resistance Surveillance Program (ARSP) of the Department of Health (DOH) (ARSP, 2023). Based on their data for *E. coli* with commonly used oral agents, the highest resistance rates were also with AMP (78%), SXT (52%), CIP (47%), and CXM (42%) (ARSP, 2023). The similarity of resistance rates between water and clinical samples is particularly notable for CIP (45.8% vs. 47%, respectively). For parenteral antibiotics, the 2022 ARSP also reported the following resistance rates for *E. coli*: CRO (38%), CAZ (27%), FEP (20%), GEN (19%), FOX (19%), TZP (18%), MEM (8.5%), IPM (8.3%), ETP (7.6%), and AMK (3.2%) (ARSP, 2023). However, the resistance rates in water isolates were significantly lower, with CRO at 6%, and 4% for CAZ, GEN, TZP, IPM, and ETP. No resistance was observed for FEP, FOX, MEM, or AMK. The higher resistance rates in clinical isolates can likely be attributed to the increased selective pressure from broad-spectrum antibiotics used in patient treatments. Regardless, MAR and MDR*E. coli* were present in water samples, accounting for 18.8% and 14.5% of the isolates, respectively (see Table 2). The high coliform and *E. coli* concentrations in the estuaries suggest that the AR bacteria likely entered the water through human and pet excretions or as a result of antibiotic pollution from sewage connected to communities and medical facilities. This is more probable than resistance emerging from naturally occurring antibiotics produced by other microorganisms (Ancheta, 2021; Larsson & Flach, 2022).

The Frequency of AR, MAR, MDR, and ESBL *E. coli*

Among the 48 isolates, 17 (35.4%) were fully susceptible to all the 16 tested antibiotics, whereas 22 (45.8%), 9 (18.8%), 7 (14.6%), 3 (6.3%) were classified as AR, MAR, MDR, and ESBL producer, respectively (see Table 2). All MAR isolates had a MAR index of ≥0.2, indicating that these *E. coli* strains likely originated from contamination sources such as hospitals or local health centers where antibiotic use is prevalent. However, no significant association was found between AR patterns and collection month ($P \geq 0.234$); see Table 3. This could be due to the similar AR profile of *E. coli* that these estuaries received between February and March (Azzam et al., 2017).

Regardless, the findings have profound implications for public health. The AR *E. coli* in Manila's estuaries raises concerns about the increased risk of waterborne infections. Many MDR and ESBL producers, which makes infections more difficult to treat, limits therapeutic options and drives up healthcare costs (Thaden et al., 2017). This is especially critical in densely populated, low-income communities near these water sources, where access to healthcare may already be limited. From an environmental management perspective, the findings study highlight the urgent need to address water contamination, which is primarily driven by untreated domestic waste, informal settlements, and hospital runoff. Immediate action is needed to improve waste management systems, such as investing in wastewater treatment facilities, and enforcing stricter regulations on sewage disposal. These actions are essential to curbing the spread of AR bacteria in natural water bodies (Ho et al., 2021; Irfan et al., 2022).

Limitations of the Study

Despite the results, the study's limited two-month sampling period (February and March 2024) may overlook seasonal variations affecting antibiotic resistance. While higher *E. coli* levels in March were linked to temperature, data from other seasons, such as the rainy season, is needed to capture year-round fluctuations (Jiang et al., 2021; Liang et al., 2020). Furthermore, the study's sampling was limited to six estuaries in Manila. This may introduce bias, as it might not reflect other estuaries in the city with different or lower pollution levels, potentially overestimating the prevalence of MDR and ESBL isolates. Local factors like density of informal settlements and hospital

Table 2: Frequencies and percentage of AR levels

Result	*Frequency (Percent, %)*				
	FS	*AR*	*MAR*	*MDR*	*ESBL*
Positive	17 (35.4)	22 (45.8)	9 (18.8)	7 (14.6)	3 (6.3)
Negative	31 (64.6)	26 (54.2)	39 (81.2)	41 (85.4)	45 (93.8)

AR, antibiotic resistant; ESBL, extended spectrum beta lactamase producer; FS, fully susceptible; MAR, multiple antibiotic resistant; MDR, multidrug resistant wastewater could skew the results, thus a broader geographic sampling would offer a more complete picture of antibiotic resistance (Quintela-Baluja et al., 2019; Wang et al., 2020; Zheng et al., 2021). Finally, sampling at shallow depths (1 to 15 cm) may not capture the full range of microbial activity, potentially missing AR bacteria at greater depths and leading to an incomplete understanding of their distribution (Fu et al., 2022; Zhang et al., 2022).

Table 3: Frequency of MAR, MDR, and ESBL *E. coli* by month of collection

Result	*Frequency per month, n = 24*		
	February	*March*	*p value*
MAR	3	6	0.461
MDR	3	4	1.000
ESBL	3	0	0.234

ESBL, extended spectrum beta lactamase producer; MAR, multiple antibiotic resistant; MDR, multidrug resistant

Conclusions

The concentration and isolation rates of MDR *E. coli* in Manila's estuaries remain critically high. Despite the enforcement of local laws, ordinances, and public awareness campaigns, the estuaries continue to deteriorate due to the direct discharge of septic waste and untreated wastewater (Bringula et al., 2015). Thus, expanding AR surveillance beyond clinical settings, through a "One Health" approach that includes estuarine waters, could provide the necessary impetus to mobilise stakeholders for more effective interventions aimed at curbing the spread of AR bacteria in these waters (Diallo et al., 2020; Şahin et al., 2021). However, the study's limited temporal and spatial scope introduces biases that affect the generalisability of its findings. Extending the study period and broadening the geographic sampling would strengthen the conclusions and provide a more comprehensive understanding of AR in these highly urbanised estuarine environments.

Acknowledgements

The authors are grateful to the Department of Medical Technology, Far Eastern University, for allowing the use of the research facility and the technical assistance of staff for this work.

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