

## Genotypic detection of $\beta$ -lactamase-producing *Escherichia coli* isolates obtained from Seven Crater Lakes of San Pablo, Laguna, Philippines

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### ABSTRACT

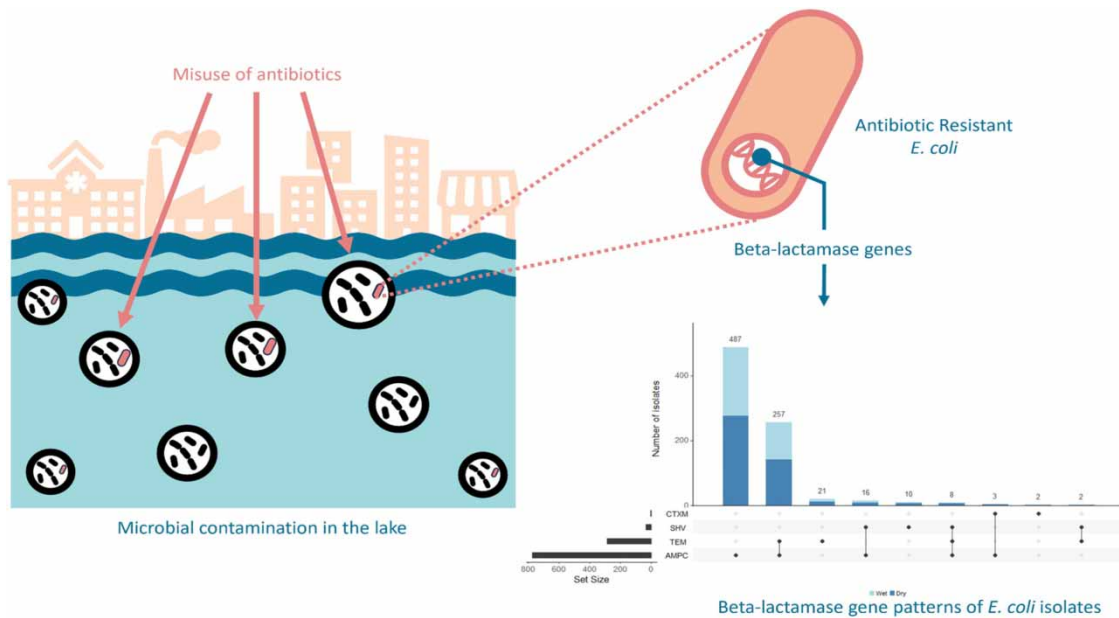
The extended-spectrum  $\beta$ -lactamase (ESBL)-producing *Escherichia coli* is becoming a global public health concern. More comprehensive surveillance of  $\beta$ -lactam resistance in *E. coli* would improve monitoring strategies and control resistance transmission in contaminated environments. This study investigated the prevalence of  $\beta$ -lactamase genes in *E. coli* isolated from the Seven Crater Lakes in San Pablo, Laguna, Philippines. Water samples from lakes were collected for the isolation of *E. coli* ( $n = 846$ ) and molecular characterization by detecting the presence of the *uidA* gene. The isolates were then tested for the presence of  $\beta$ -lactamase genes using PCR. Among the screened genes, *bla*<sub>AmpC</sub> was the most dominant (91%). Other  $\beta$ -lactamase genes such as *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>CTXM</sub> were also detected with percentage occurrence of 34, 5, and 1%, respectively. Multiple genes within individual isolates were also observed, wherein *bla*<sub>TEM/AmpC</sub> was the most prevalent gene combination. Moreover, a significant negative correlation between *bla*<sub>AmpC</sub> with *bla*<sub>SHV</sub> and *bla*<sub>CTXM</sub> was depicted in this study. Overall, these findings demonstrate the presence of  $\beta$ -lactamase genes in *E. coli* in the Seven Crater Lakes of San Pablo and can be used in developing effective strategies to control antibiotic resistance in environmental waters.

**Key words:** AmpC, antibiotic resistance, extended-spectrum  $\beta$ -lactamase, *Escherichia coli*, Laguna, Philippines

### HIGHLIGHTS

- *bla*<sub>AmpC</sub> was the predominant gene detected among the five screened  $\beta$ -lactamase genes.
- A significant association was detected between *bla*<sub>AmpC</sub> with *bla*<sub>SHV</sub> and *bla*<sub>CTXM</sub>.
- *bla*<sub>KPC</sub> was not detected in any *E. coli* isolates from the lakes.
- Seasonal variations have an effect on the fecal coliform counts and occurrence of  $\beta$ -lactamase-resistant *E. coli* isolates.

## GRAPHICAL ABSTRACT



## 1. INTRODUCTION

The emergence of antibiotic resistance creates a major threat to animals, people, and the environment (Walsh & Duffy 2013; Zhu *et al.* 2019). Mutations that confer antibiotic resistance can be transmitted vertically and through horizontal gene transfer using mobile genetic elements such as plasmids and transposons (Fletcher 2015). Microbial contaminants can be transferred into various environments by agricultural practices, wastewater effluents, soil erosion, soil leaching, and groundwater contamination (Yavuz Corapcioglu & Haridas 1985). Animal wastes, a reservoir of these bacteria, can also contribute to the transmission. These antibiotic-resistant bacteria can infect or be ingested by humans and animals, which spread among them through food and the environment. Other factors independent of human activities, like nutrient concentration, heavy metal concentration, and other environmental parameters, may also contribute to the presence of antibiotic resistance genes (ARGs) (Chen *et al.* 2019; Cycoń *et al.* 2019; Song *et al.* 2020).

In recent years, antibiotic-resistant *Escherichia coli*, specifically extended-spectrum  $\beta$ -lactamase (ESBL)-producing *E. coli*, has gained attention and is now considered a serious threat to human health (World Health Organization 2017). ESBLs are  $\beta$ -lactamases with an extended action range formed from mutations or passed through horizontal gene transfer. They are present in various classes, like TEM, SHV, and CTXM, which occasionally show a moderately low homology and are resistant against cephalosporin (Paterson & Bonomo 2005). Other classes of  $\beta$ -lactamases include AmpC and *Klebsiella pneumoniae* carbapenemase, which mostly confer resistance against penicillin and carbapenem, respectively (Thomson 2010). All these enzymes are generally plasmid-coded and hence more effectively transmissible. They can hydrolyze  $\beta$ -lactam antibiotics, thereby bringing protection and resistance against them (Reinthalder *et al.* 2010).  $\beta$ -lactamase-producing *E. coli* may have adapted the genes for this trait mainly due to the simplicity of their transmission among humans and animals through the fecal-oral route and their ability to live inside the intestines of their hosts, allowing their close interaction with other microorganisms behaving as either donor or recipient of resistant genes to adjacent bacteria (Poirel *et al.* 2018).

Laguna has been considered a rapidly urbanizing province in the Philippines. With this, green spaces encompassing the province are exposed to land usage, bringing about numerous environmental problems, including solid waste and microbial contamination (Quintal *et al.* 2018). The Seven Crater Lakes in San Pablo, Laguna, namely Bunot, Calibato, Mohicap, Palakpak, Pandin, Sampaloc, and Yambo, have comparable issues. These lakes supply locals with food, sustenance, transportation, and recreation, and are important in agriculture and aquaculture (Paller *et al.* 2021). Previous studies have revealed microbial contamination in these freshwater bodies. An investigation from Gacad & Briones (2020) showed the

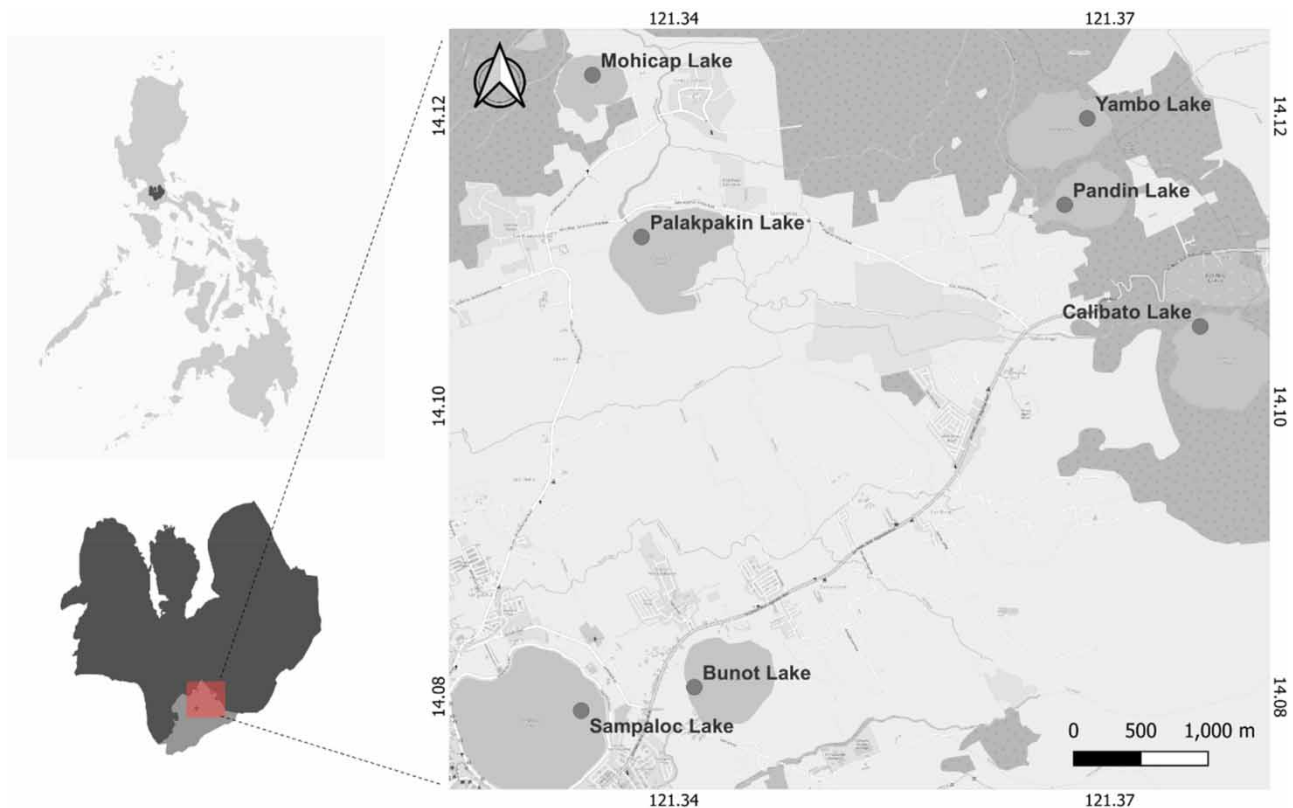
presence of pathogenic bacteria *Aeromonas veronii* and *Plesiomonas shigelloides* in Sampaloc Lake. Moreover, waterborne protozoan pathogens, like *Acanthamoeba* spp., *Cryptosporidium* spp., and *Giardia* spp., were also detected in these lakes (Ballares *et al.* 2020; Masangkay *et al.* 2020). Although the lakes are consistently being monitored by the Laguna Lake Development Agency (LLDA), little is known about the presence of *E. coli*-harboring  $\beta$ -lactamase genes in these lakes.

Representative genes of  $\beta$ -lactamases, namely, *bla*<sub>CTXM</sub>, *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>AmpC</sub>, and *bla*<sub>KPC</sub>, were utilized to examine the resistance gene patterns of *E. coli* isolates across water samples in San Pablo's seven lakes. Specifically, the study aimed to determine the genotypic profile of *E. coli*-harboring  $\beta$ -lactamase genes, display the gene patterns, determine the association between these genes, and evaluate correlation between environmental factors and gene frequencies. Accordingly, this investigation could address information gaps in improving regulation and guidelines and giving human implications like expanding public awareness and anticipating serious risks of infections.

## 2. MATERIALS AND METHODS

### 2.1. Study site and sample collection

Water samples were collected from the Seven Crater Lakes of San Pablo, Laguna, namely, Bunot, Calibato, Mohicap, Palakpakin, Pandin, Sampaloc, and Yambo, as visualized in Figure 1 and coordinates listed in Table 1. Two-liter water samples were obtained from a 20-cm depth in each of the seven lakes and stored in sterile wide-mouth Nalgene bottles (ThermoFisher Scientific, Rochester, NY, USA). The sites were based on water quality monitoring and sampling sites operated by the LLDA. Physicochemical and microbiological parameters, which were also based on the water quality guidelines of the monitoring agency, were immediately measured following the sample collection. Samples were collected between October 2022 and June 2023, providing representative isolates during wet and dry seasons. After collection, samples were stored in cold boxes and immediately transported to the laboratory for processing.



**Figure 1** | Map showing the Seven Crater Lakes of San Pablo, Laguna, Philippines. The inset shows the sampling sites relative to San Pablo City in Laguna province (R Core Team 2022).

**Table 1** | Location of study sites and count of confirmed *E. coli* isolates

Sites	Coordinates		Number of confirmed <i>E. coli</i> isolates		
	Latitude	Longitude	Wet	Dry	Total
Bunot	14.080667	121.341417	36	77	113
Calibato	14.105503	121.376239	94	63	157
Mohicap	14.122822	121.334401	18	112	130
Palakpakin	14.111667	121.337778	59	53	112
Pandin	14.113878	121.366912	45	37	82
Sampaloc	14.079058	121.333634	78	100	178
Yambo	14.079058	121.333634	31	43	74
Total			361	485	846

## 2.2. Isolation and identification of thermotolerant *Escherichia coli*

Five milliliters of the water sample were serially diluted from  $10^{-1}$  to  $10^{-5}$  in a 45-mL 0.9% sodium chloride solution before filtering through a membrane filter (0.45  $\mu\text{m}$  pore size; Pall Corp., USA) using a vacuum pump. These were done in triplicates. The membranes were then placed on membrane-thermotolerant *E. coli* (mTEC; TM Media) agar, incubated at 37 °C for 2 h, and then transferred to 44.5 °C for 18–24-h incubation. Yellow colonies, presumed as *E. coli*, were then picked and confirmed on eosin methylene blue agar (EMBA; BD BBL, USA) plates and incubated at 35 °C for 24 h. Dark blue to purple colonies with metallic green sheen were confirmed *E. coli* isolates and then subjected to streaking for purification and further molecular confirmation. Isolates were stored at –21 °C in tryptic soy broth (TSB; BD BBL, USA) with 20% (v/v) glycerol.

For molecular confirmation, selected *E. coli* isolates were inoculated into 1 mL TSB and were then incubated at 37 °C for 18–24 h. The DNA of *E. coli* isolates was extracted using the boil-lysis method (Garcia *et al.* 2015). A PCR assay was used to detect the 75-bp *uidA* gene (i.e.,  $\beta$ -glucuronidase gene) in all extracts using the primers ECN1254F (5'-GCAAGGTGCACGG-GAATATT-3') and ECN1328R (5'-CAGGTGATCGGACGCGT-3') (Labrador *et al.* 2020). The reaction mixture consisted of 1X GoTaq<sup>®</sup> Green Master Mix (Promega, USA), 0.5  $\mu\text{M}$  each of forward and reverse primers, 1  $\mu\text{L}$  DNA template, and nuclease-free water for a total volume of 10  $\mu\text{L}$ . The PCR for confirmation of *E. coli* was performed using the method of Takahashi *et al.* (2009) with initial denaturation at 98 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 63 °C for 1 min, extension at 72 °C for 1 min, and one cycle of final extension at 72 °C for 5 min. A no-template control was included in every run. Isolates with amplicons that met the expected band size were selected to detect ARGs. The number of confirmed *E. coli* isolates per lake and season is shown in Table 1.

## 2.3. Detection of ARGs

PCR multiplex assay was used to determine the presence of three  $\beta$ -lactam ARGs, namely, *bla*<sub>CTXM</sub>, *bla*<sub>TEM</sub>, and *bla*<sub>SHV</sub>, while a singleplex assay was utilized for *bla*<sub>AmpC</sub> and *bla*<sub>KPC</sub> in all confirmed 846 thermotolerant *E. coli*. The reaction mixture components and PCR conditions for each assay were adapted from Monstein *et al.* (2007) for the multiplex assay, Feria *et al.* (2002) for *bla*<sub>AmpC</sub>, and Poirel *et al.* (2011) for *bla*<sub>KPC</sub> detection. The identity of the PCR amplicons generated using primers *bla*<sub>CTXM</sub>, *bla*<sub>TEM</sub>, and *bla*<sub>SHV</sub> was already confirmed through DNA sequencing by Monstein *et al.* (2007), while amplicons from *bla*<sub>AmpC</sub> and *bla*<sub>KPC</sub> were also validated by Feria *et al.* (2002) and Naas *et al.* (2005), respectively.

Table 2 summarizes the primer sequences and corresponding amplicon sizes for each gene. Positive and no-template controls were employed in all runs. Amplicons were then characterized using agarose gel electrophoresis. The 100-bp DNA ladder (Hyperladder<sup>™</sup> Bioline), controls, and the amplicons were loaded into 1.5% (w/v) agarose gel stained with SYBR<sup>®</sup> Safe DNA gel stain (Invitrogen, USA). The loaded samples were subjected to an electrophoresis system containing 1 $\times$  TAE buffer at 280 V for 30 min. The resulting DNA bands were then viewed under UV illumination (Bio-Print ST4, Vilber Lourmat, UK).

## 2.4. Data analyses

All statistical analyses were conducted using R v 4.1.3 (R Core Team 2022). Spearman's rank correlation test was used to test for association across  $\beta$ -lactamase gene resistance of *E. coli* isolates as well as to examine the correlation between

**Table 2** | Primer sets for the detection of  $\beta$ -lactam ARGs used in the study

Target gene	Primer sequence	Amplicon (bp)	Positive control	References
<i>bla</i> <sub>TEM</sub>	TCGCCGCATACACTATTCTCAGAATGA ACGCTCACCGGCTCCAGATTTAT	445	<i>Salmonella</i> sp.	Monstein <i>et al.</i> (2007)
<i>bla</i> <sub>CTXM</sub>	ATGTGCAGYACCAGTAARGTKATGGC TGGGTRAARTARGTSACCAGAAYCAGC	593		
<i>bla</i> <sub>SHV</sub>	ATGCGTTATATTCGCCTGTG TGCTTTGTTATTCGGGCCAA	745	<i>Klebsiella pneumoniae</i>	
<i>bla</i> <sub>AmpC</sub>	CCCCGCTTATAGAGCAACAA TCAATGGTCGACTTCACACC	634	<i>E. coli</i>	Feria <i>et al.</i> (2002)
<i>bla</i> <sub>KPC</sub>	CGTCTAGTTCTGCTGTCTTG CTTGTATCCTTGTAGGCC	798	<i>K. pneumoniae</i>	Poirel <i>et al.</i> (2011)

environmental parameters and frequency of *E. coli*-harboring  $\beta$ -lactamase resistance genes. Meanwhile, the UpsetR package was applied to visualize intersections and patterns in the number of isolates with associated  $\beta$ -lactamase genes (Gehlenborg 2019). Finally, the Wilcoxon rank sum test was utilized to determine significant differences between seasons in terms of the frequency of *E. coli* isolates and water quality parameters in seven lakes.

### 3. RESULTS

#### 3.1. Environmental parameters of San Pablo Lakes

As shown in Table 3, water quality parameters such as pH, total suspended solids (TSS), biochemical oxygen demand (BOD), dissolved oxygen (DO), ammonia, nitrate, inorganic phosphate, temperature, and fecal coliform counts were measured using different analyses (Supplementary material, Table S1). Unfortunately, not all environmental parameters of the lakes tested met the water parameter guidelines classification C by the Department of Environment and Natural Resources (DENR) (Supplementary material, Table S1).

Inorganic phosphate at Bunot in the dry season and Calibato, Mohicap, and Sampaloc in both seasons have higher values as compared to the standard upper limit of 0.5 mg/L. Lakes Calibato and Mohicap in the wet season also exceeded the upper limit value for BOD, which is 7 mg/L. Except for lakes Pandin and Yambo, the ammonia levels for other lakes exceeded the upper limit value, which is 0.05 mg/L. It must also be noted that Lake Mohicap in the dry season and Palakpakin during the

**Table 3** | Physicochemical and bacteriological parameters of San Pablo lakes between dry and wet seasons

Season	Site	pH	TSS (mg/L)	BOD (mg/L)	DO (mg/L)	Ammonia (mg/L)	Nitrate (mg/L)	Inorganic phosphate (mg/L)	Temperature (°C)	Fecal coliform (MPN/100 mL) <sup>a</sup>
Dry	Bunot	7.3	3	5	5.6	<b>2.05</b>	0.05	<b>0.9</b>	26	<b>330</b>
	Calibato	7.1	6	5	9.7	<b>1.44</b>	0.12	<b>0.94</b>	28	110
	Mohicap	7.3	3	7	<b>4.6</b>	<b>1</b>	0.36	<b>1.47</b>	29	45
	Palakpakin	7.4	2	4	5	<b>0.17</b>	0.46	0.35	28	20
	Pandin	7.5	4	1	7.4	0.04	0.05	0.03	27	40
	Sampaloc	7.3	9	3	5.9	<b>0.99</b>	0.31	<b>1.5</b>	29	<b>490</b>
	Yambo	7.5	2	0.5	7.4	0.03	0.05	0.02	29	<b>330</b>
Wet	Bunot	7.2	7	6	8.2	<b>2.1</b>	0.03	0.5	27	<b>300</b>
	Calibato	7.1	2	<b>9</b>	10.4	<b>2.05</b>	0.32	<b>0.74</b>	29	78
	Mohicap	7.2	7	<b>8</b>	7.2	<b>2.19</b>	0.03	<b>0.54</b>	30	45
	Palakpakin	7.5	6	3	<b>3.2</b>	<b>0.25</b>	0.19	0.28	29	<b>260</b>
	Pandin	7.2	1	2	7.6	0.005	0.05	0.04	28	140
	Sampaloc	7.5	3	6	9	1	0.18	1.46	30	1,700
	Yambo	7.5	2	0.5	7.4	0.03	0.05	0.02	29	330

<sup>a</sup>Comparison of parameters between two seasons was done with the Wilcoxon rank sum test ( $p$  value  $\leq 0.05$ ).

Bolded values signify values that were higher than the environmental guidelines.

wet season were below the lower limit value of DO, which is 5 mg/L. Moreover, fecal coliform at Palakpakin in the wet season as well as Bunot, Sampaloc, and Yambo in both seasons also did not meet the standard values. These suggest that chemical and fecal pollution occurs in some lakes. Pandin Lake, which is considered the most pristine among all lakes, is the only site that passed all the standard water quality guidelines in both seasons.

Wilcoxon rank sum test was utilized to find out if there is significant difference between the sites and seasons in terms of the environmental parameters. The results showed no significant difference between sites; however, a statistically significant difference in fecal coliform counts between dry and wet seasons was observed ( $p = 0.046$ ) suggesting that a higher level of fecal pollution occurs during the wet season.

### 3.2. *bla*<sub>AmpC</sub> is the predominant $\beta$ -lactamase gene detected

Resistance patterns were recorded across four  $\beta$ -lactamase genes (Figure 2). Among the 846 isolates, 487 harbored *bla*<sub>AmpC</sub> alone, 21 isolates were detected with *bla*<sub>TEM</sub>, 10 isolates harbored *bla*<sub>SHV</sub>, and two isolates were detected with *bla*<sub>CTXM</sub>. No *bla*<sub>KPC</sub> was detected in the isolates. For isolates harboring two  $\beta$ -lactamase genes, 257 isolates possessed *bla*<sub>TEM/AmpC</sub>, 16 were detected with *bla*<sub>SHV/AmpC</sub> both in the wet and dry seasons, three harbored *bla*<sub>CTXM/AmpC</sub> during the dry season, and two were detected with *bla*<sub>TEM/SHV</sub> during the dry season. However, no isolates were detected with *bla*<sub>TEM/CTXM</sub> and *bla*<sub>SHV/CTXM</sub> gene combinations. Finally, eight isolates harbor *bla*<sub>SHV/TEM/AmpC</sub>. A high prevalence of *bla*<sub>AmpC</sub> and a combination of *bla*<sub>AmpC/TEM</sub> were observed in most *E. coli* isolates among the various  $\beta$ -lactamase resistance patterns.

The presence of five molecular determinants was tested on all *E. coli* isolates. Of the 846 isolates, 93% (336/361) and 90% (435/485) of the isolates harbored *bla*<sub>AmpC</sub> during wet and dry seasons, respectively, showing the highest percent occurrence among the screened  $\beta$ -lactamase genes. This was followed by *bla*<sub>TEM</sub>-producing *E. coli* isolates with a percent occurrence of 35% (126/361) during the wet season and 33% (162/485) during the dry season. Both genes have representative *E. coli* isolates in each lake over the two seasons. Meanwhile, *bla*<sub>SHV</sub> was not detected in the isolates in Calibato Lake but was highest in Pandin with a 17% occurrence. Conversely, isolates with *bla*<sub>AmpC</sub> have the lowest percent occurrence of 84% in Pandin Lake. Furthermore, *bla*<sub>CTXM</sub> in *E. coli* isolates was detected in Palakpakin and Sampaloc during the dry season only, while no *bla*<sub>KPC</sub> was detected in all isolates during both seasons (Table 4). As no *bla*<sub>KPC</sub> was detected in the isolates, this gene was excluded in the succeeding analyses. Using the Wilcoxon rank sum test, a significant difference ( $p = 3.34 \times 10^{-10}$ ) was observed

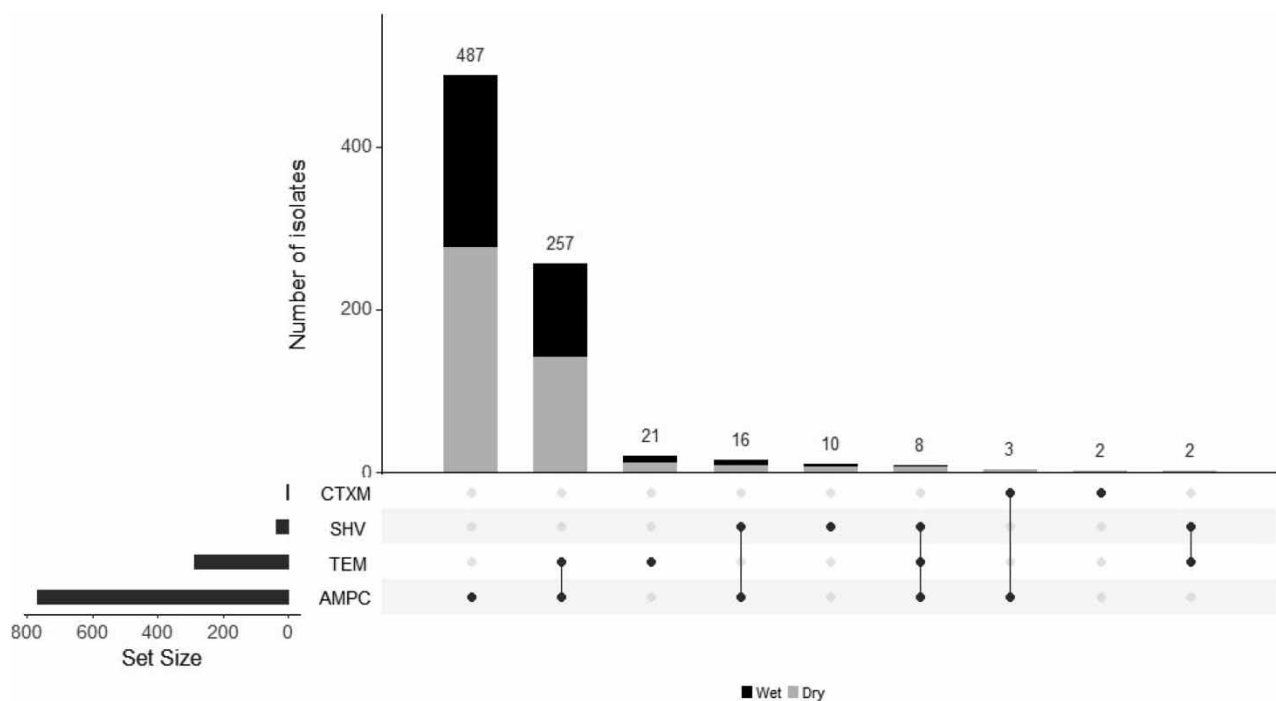


Figure 2 |  $\beta$ -Lactamase gene patterns of *E. coli* isolates between wet and dry seasons.

**Table 4** | Number and percentage occurrence of  $\beta$ -lactamase genes in *E. coli* isolates from Seven Crater Lakes over two seasons

Site	Number (%) of $\beta$ -lactamase gene-harboring <i>E. coli</i> isolates				
	<i>bla</i> <sub>TEM</sub>	<i>bla</i> <sub>CTXM</sub>	<i>bla</i> <sub>SHV</sub>	<i>bla</i> <sub>AmpC</sub>	<i>bla</i> <sub>KPC</sub>
Bunot ( <i>n</i> = 113)	39 (35%)	0 (0)	4 (5%)	104 (92%)	0 (0)
Calibato ( <i>n</i> = 157)	83 (53%)	0 (0)	0 (0)	145 (92%)	0 (0)
Mohicap ( <i>n</i> = 130)	58 (45%)	0 (0)	7 (5%)	120 (92%)	0 (0)
Palakpakin ( <i>n</i> = 112)	39 (35%)	3 (3%)	4 (4%)	102 (91%)	0 (0)
Pandin ( <i>n</i> = 82)	22 (27%)	0 (0)	14 (17%)	69 (84%)	0 (0)
Sampaloc ( <i>n</i> = 178)	31 (17%)	2 (1%)	6 (3%)	160 (90%)	0 (0)
Yambo ( <i>n</i> = 74)	16 (22%)	0 (0)	1 (1%)	71 (96%)	0 (0)
Seasons <sup>a</sup>					
Wet ( <i>n</i> = 361)	126 (35%)	0 (0)	12 (3%)	336 (93%)	0 (0)
Dry ( <i>n</i> = 485)	162 (33%)	5 (1%)	24 (5%)	435 (90%)	0 (0)
Total ( <i>n</i> = 846)	288 (34%)	5 (1%)	36 (4%)	771 (91%)	0 (0)

<sup>a</sup>Comparison of frequencies between two seasons was done with the Wilcoxon rank sum test ( $p$  value  $\leq 0.05$ ).

between the two seasons, with the wet season having a higher frequency of  $\beta$ -lactamase-harboring *E. coli* isolates than the dry season. This suggests that the type of season significantly affects the occurrence of these isolates.

### 3.3. Association between $\beta$ -lactamase genes in *E. coli* isolates

To determine correlations between *E. coli*-harboring  $\beta$ -lactamase genes, Spearman's rank correlation test was utilized (Table 5). Significant associations were displayed between *bla*<sub>AmpC</sub> and *bla*<sub>CTXM</sub> ( $p = 0.014$ ), including *bla*<sub>AmpC</sub> and *bla*<sub>SHV</sub> ( $p = 1.07 \times 10^{-7}$ ). Specifically, these genes have a negative correlation as displayed by their negative rho values. This indicates that for a large number of isolates harboring *bla*<sub>AmpC</sub>, few isolates of *bla*<sub>CTXM</sub> and *bla*<sub>SHV</sub> have been observed. Additionally, *bla*<sub>SHV</sub>-*bla*<sub>CTXM</sub>, *bla*<sub>TEM</sub>-*bla*<sub>CTXM</sub>, and *bla*<sub>TEM</sub>-*bla*<sub>SHV</sub> were negatively correlated but were not statistically significant. Moreover, although no significant association was displayed between *bla*<sub>TEM</sub> and *bla*<sub>AmpC</sub>, it is notable that they are positively correlated.

### 3.4. Correlation between gene frequency and water quality parameters

Spearman's rank correlation test was also used to examine correlations between  $\beta$ -lactamase gene frequencies and environmental parameters (Table 6). The results displayed that the pH, TSS, BOD, DO, ammonia, inorganic phosphate, temperature, and fecal coliform did not show any significant correlation with the occurrence of isolates harboring  $\beta$ -lactamase genes. However, nitrate showed a significant correlation with the frequency of these resistant isolates ( $p = 0.038$ ).

## 4. DISCUSSION

Environmental waters, such as rivers and lakes, are utilized for commercial transport, waste disposal, livelihood, and recreation. These are also surrounded by urban lands that often undergo industrialization or agricultural farms that can greatly affect the waters' structure, functionality, and quality (Loucks & van Beek 2017). Due to this frequent land use change,

**Table 5** | Spearman's rank correlation test shows association of resistant *E. coli* isolates across four  $\beta$ -lactamase genes

	<i>bla</i> <sub>TEM</sub>	<i>bla</i> <sub>CTXM</sub>	<i>bla</i> <sub>SHV</sub>	<i>bla</i> <sub>AmpC</sub>
<i>bla</i> <sub>TEM</sub>	–			
<i>bla</i> <sub>CTXM</sub>	–0.055	–		
<i>bla</i> <sub>SHV</sub>	–0.028	–0.016	–	
<i>bla</i> <sub>AmpC</sub>	0.022	–0.085*	–0.18*	–

\*Significant at  $p \leq 0.05$ .

**Table 6** | Spearman's rank correlation coefficients between frequency of resistant *E. coli* isolates and water quality parameters

Parameters	Frequency of $\beta$ -lactamase-harboring <i>E. coli</i> isolates
pH	0.036
TSS (mg/L)	0.036
BOD (mg/L)	-0.036
DO (mg/L)	-0.39
Ammonia (mg/L)	-0.36
Nitrate (mg/L)	0.13*
Inorganic phosphate (mg/L)	-0.11
Temperature(°C)	-0.16
Fecal coliform (MPN/100 mL)	-0.57

\*Significant at  $p \leq 0.05$ .

agricultural and urban land use have associated these water bodies with antibiotic resistance transmission and reservoirs (Nnadozie & Odume 2019; Xu *et al.* 2020; Salvador-Membreve & Rivera 2021).  $\beta$ -lactamase-producing *E. coli* has gained attention worldwide due to its public health threat and is recognized as one of the multidrug-resistant bacteria associated with serious infections (World Health Organization 2017). Thus, understanding the role and increasing the information of  $\beta$ -lactamase genes through surveillance and profiling is necessary for management efforts in these systems. In this study, *E. coli* isolates from the Seven Crater Lakes of San Pablo were characterized for their presence of  $\beta$ -lactamase genes to determine the extent of threat of these genes in the contaminated water body.

Organic, inorganic, and fecal pollution were observed in most lakes of San Pablo. Specifically, ammonia displayed high levels in all lakes except for Pandin and Yambo, while inorganic phosphate levels were higher in lakes Bunot, Calibato, Mohicap, and Sampaloc for both seasons. The data suggest that lakes Pandin and Yambo are oligotrophic, which could be due to good management and development plans of the lakes for recreational purposes. The results also displayed a significantly higher influx of fecal coliform during the wet season. A greater amount of rainfall increases fecal coliform counts due to the pollution brought by surface runoff. The study of Hernandez *et al.* (2020) and Hill *et al.* (2006) also depicted a higher total coliform count in the wet than in the dry season as fecal pollutants are being washed into the environmental waters by the rain. Except for BOD, DO, ammonia, inorganic phosphate, and fecal coliform, other environmental parameters measured in the study, such as pH, TSS, nitrate, and temperature, satisfy the water quality guidelines of the DENR.

In this study, 846 *E. coli* isolates have been detected from the Seven Lakes of San Pablo. The highest percent occurrence of ARGs detected was 91%, attributed to the isolates possessing *bla*<sub>AmpC</sub>, followed by those with *bla*<sub>TEM</sub> (34%), *bla*<sub>SHV</sub> (4%), and *bla*<sub>CTXM</sub> (1%). It is also noteworthy that *bla*<sub>CTXM</sub> was only detected in five isolates from Palakpakin and Sampaloc Lake during the dry season. It is possible that *bla*<sub>CTXM</sub> was more spatially variable in the dry than in the wet season, and the proximity of wastes from domestic houses and establishments surrounding the lake can be a factor for this occurrence (Knapp *et al.* 2012). The gene *bla*<sub>SHV</sub> has the highest frequency in *E. coli* detected from Pandin Lake, while *bla*<sub>AmpC</sub> had the lowest frequency in the same lake. Meanwhile, the gene *bla*<sub>KPC</sub> was not detected in all isolates. The prevalence of *bla*<sub>AmpC</sub> and *bla*<sub>TEM</sub> is similar to most reported studies on *Enterobacteriaceae* and ARG detection in environmental waters (Mohd Khari *et al.* 2016; Nzima *et al.* 2020; Salvador-Membreve & Rivera 2021; Zieliński *et al.* 2021). However, this is contrary to the studies of Mahmud *et al.* (2020) and Zaatout *et al.* (2021) where *bla*<sub>CTXM</sub> is the predominant gene detected among ESBL-producing *E. coli*. AmpC gene is often expressed in higher amounts and is transmitted faster to other bacteria explaining its high occurrence in *E. coli* isolates (Mohd Khari *et al.* 2016).

Previous studies suggest that ESBL genes differ between geographical areas (Gundran *et al.* 2019). Thus, it is important to perform wider surveillance to determine the level of dissemination of these genes in environmental waters in the Philippines. Meanwhile, Stoesser *et al.* (2017) showed that other variants of *bla*<sub>KPC</sub>, originating from *K. pneumoniae*, remain rare in *E. coli* depending on the geographical location, thus explaining the absence of the gene from this study. Cases of *bla*<sub>KPC</sub>-harboring *E. coli* due to plasmid-mediated transfer have been reported but are limited to some countries in North and South America and Asia, like China, and have not been observed yet in the Philippines (Robledo *et al.* 2011; Luo *et al.* 2014; Kazmierczak *et al.* 2016).



Regarding gene patterns, the co-occurrence of *bla*<sub>AmpC</sub> and *bla*<sub>TEM</sub> in individual *E. coli* isolates was the most dominant in all seven lakes, while few isolates have combinations of *bla*<sub>AmpC</sub> with *bla*<sub>SHV</sub> and *bla*<sub>CTXM</sub>. This finding contradicts the study of Shahid *et al.* (2012), where *bla*<sub>CTXM</sub> had a higher occurrence than *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> when representative AmpC genes were present. Geographical location might be a factor in these differences. However, simultaneous occurrences of ESBL genes with *bla*<sub>AmpC</sub> gene in individual isolates can be alarming as these can cause broad-spectrum antibiotic resistance, and physiological imbalances in a bacterial cell could even lead to the development of carbapenem resistance (Shahid *et al.* 2012).

Multiple genes comprising three  $\beta$ -lactamase genes (*bla*<sub>AmpC</sub>, *bla*<sub>TEM</sub>, and *bla*<sub>SHV</sub>) on one of the eight isolates have also been detected in Pandin Lake during the wet season while seven isolates were observed in lakes Mohicap and Sampaloc during the dry season. A few studies have reported the coexistence of  $\beta$ -lactamase genes within the same *E. coli* isolates obtained from environmental waters (Liu *et al.* 2018; Ali *et al.* 2021). It is noteworthy that a significant difference was observed between the two seasons in terms of the occurrence of *E. coli* isolates. This may be explained by the role of high precipitation rates in increasing the frequency of agricultural runoffs, thereby increasing the concentration of microbiological contaminants in the water. Thus, seasonal variation can be a factor in multiple gene resistance in *E. coli*. This is similar to the study of Knapp *et al.* (2012), where ARG concentration was higher in the wet season than the dry season due to higher flows downstream. Although the co-occurrence of three  $\beta$ -lactamase genes in the lake is low, multiple resistance genes could still result in a contained resistance to  $\beta$ -lactamases over time.

Several studies have revealed the associations between ARGs and environmental parameters such as pH, ammonia, nitrate, orthophosphate, phosphorus, nitrogen, organic carbon, and total dissolved solids (Staley *et al.* 2015; Zhou *et al.* 2017). This study examined analyses of the association between water quality factors and the frequency of  $\beta$ -lactamase-harboring isolates. The frequency of the isolates harboring these genes had a strong positive correlation with nitrate, which corresponds with a study in eastern China wherein total dissolved nitrogen was significantly associated with the total ARGs present in the samples (Zhou *et al.* 2017). Under high organic or inorganic levels, isolates carrying genetic resistance elements had a substantially greater chance of horizontal transmission than susceptible isolates, resulting in increased spread of resistant genes (Kohyama & Suzuki 2019). No correlations were found among other parameters. This might indicate that *E. coli* encoding  $\beta$ -lactamase genes were not susceptible to environmental factors like pH, temperature, TSS, BOD, DO, ammonia, and phosphate and that other parameters must be taken into consideration (Li *et al.* 2020).

The correlation of resistance genes is often a result of co-selection, and this phenomenon is one of the reasons why it is challenging to reverse antibiotic resistance. Co-selection of genes happens when one antibiotic is enough to maintain the resistance mechanisms of ARGs linked in the same plasmid. Thus, understanding the association between these genes is important in making the challenge of treating and managing antibiotic resistance lesser and easier (Kpoda *et al.* 2018; Mazhar *et al.* 2021). This study observed a positive correlation between *bla*<sub>AmpC</sub> with *bla*<sub>TEM</sub> and a negative correlation between *bla*<sub>AmpC</sub> with *bla*<sub>SHV</sub> and *bla*<sub>CTXM</sub> among *E. coli* isolates. Studies have shown a strong association between *bla*<sub>AmpC</sub> and class A ESBLs as isolates harboring the *bla*<sub>AmpC</sub> gene has also carried ESBL genes in different combinations (Shahid *et al.* 2012). The correlation of these genes might be a result of co-selection.

Conversely, no significant association was shown in this study between *bla*<sub>TEM</sub> and *bla*<sub>CTXM</sub>, *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub>, and *bla*<sub>CTXM</sub> and *bla*<sub>SHV</sub>. With this, further information and data are needed to assess the association between these genes. Contamination in the Seven Crater Lakes of San Pablo with  $\beta$ -lactamase-producing *E. coli* could be due to open defecation from nearby houses and inappropriate wastewater management in industries, hospitals, and farms. Overall, the study has displayed that environmental *E. coli* can present public health issues by simultaneously carrying these genes.

## 5. CONCLUSION

The study's findings suggest that environmental waters examined herein could present as a reservoir for exposure and dissemination of ESBL and AmpC genes, posing a major risk to human health. The patterns and co-occurrence of these genes in *E. coli* are alarming. This is a pioneer study to report the presence of  $\beta$ -lactamase genes in bacteria from the Seven Crater Lakes of San Pablo. Seasonality had a significant effect on the occurrence of resistant *E. coli* isolates and fecal coliforms. High precipitation increases microbiological contaminants in environmental waters thereby also increasing the chance to observe isolates harboring resistant genes and fecal coliforms. One environmental water parameter like nitrate strongly correlated with *E. coli* isolates-harboring resistant genes. High nutrient concentration increases the chance of horizontal gene

transfer between isolates, thereby increasing the probability of spreading ARGs. A positive correlation between *bla*<sub>AmpC</sub> with *bla*<sub>TEM</sub> and a negative correlation between *bla*<sub>AmpC</sub> with *bla*<sub>SHV</sub> and *bla*<sub>CTXM</sub> among *E. coli* isolates were also observed. AmpC gene is often associated with faster transmission and the co-occurrences of ESBL genes with *bla*<sub>AmpC</sub> gene can pose a risk by causing broad-spectrum antibiotic resistance. Further monitoring studies in the Philippines are needed to assess the emergence and spread of multiple  $\beta$ -lactamase gene-resistant *E. coli* in the environmental waters. Moreover, strong control and management strategies are recommended to prevent the increasing antibiotic pollution, which drives more antibiotic-resistant bacteria to thrive in the environmental waters.

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## DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

## CONFLICT OF INTEREST

The authors declare there is no conflict.

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