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**Research Paper**

# Predominance of *bla*$_{TEM}$ and *tetA* genes in antibiotic-resistant *Escherichia coli* isolates from Laguna Lake, Philippines

Daile Meek Salvador-Membreve[a,b] and Windell L. Rivera[a,*]

[a] Pathogen-Host-Environment Interactions Research Laboratory, Institute of Biology, College of Science, University of the Philippines Diliman, Quezon City 1101, Philippines
[b] Biology Department, College of Science, Bicol University, Legazpi City 4500, Philippines
*Corresponding author. E-mail: wlrivera@science.upd.edu.ph

WLR, 0000-0002-4659-6132

## ABSTRACT

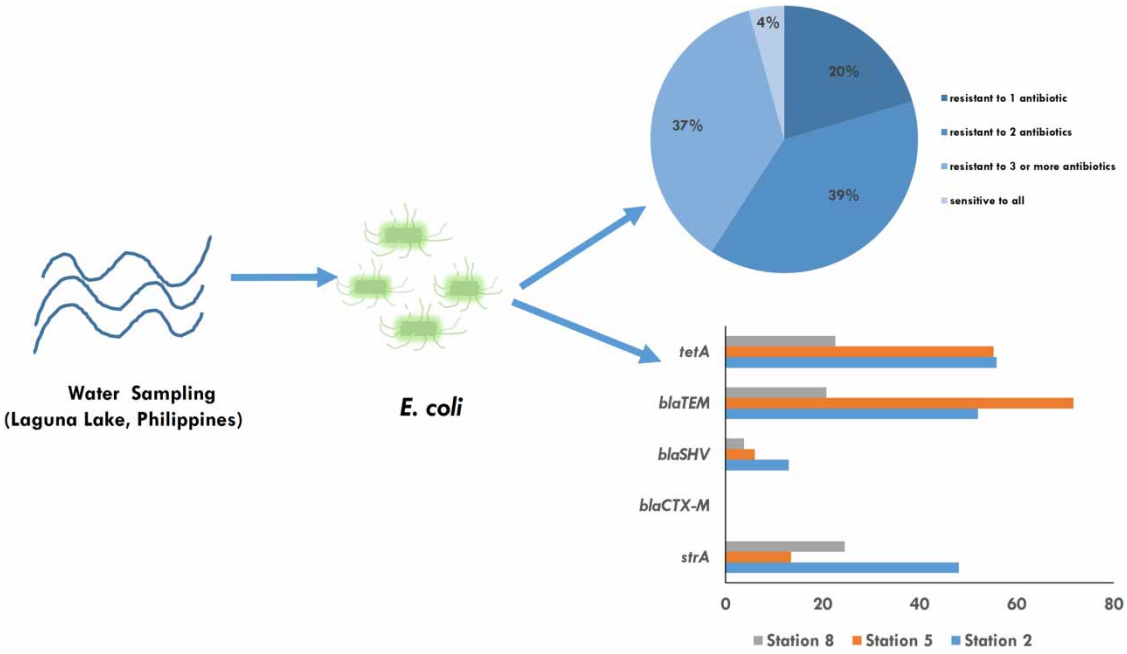
Lakes are one of the sinks of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs); however, information on ARB and ARGs in lakes in the Philippines is scarce. In this study, *Escherichia coli* was isolated from the largest freshwater lake in the Philippines, Laguna Lake, to detect antibiotic resistance and the presence of ARGs. Broth microdilution assay (BMA) and molecular identification of five environmentally prevalent ARGs (*strA*, *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$, and *tetA*) were performed. The majority (75.70%) of the isolates harbored at least one of the targeted antibiotic genes. Multiplex PCR detected about 49.07% of the isolates had genes for extended-spectrum $\beta$-lactamases (ESBL), which were mostly represented by *bla*$_{TEM}$ (47.66%). The genes *strA* and *tetA* were observed in this study with detection frequencies of 29.91 and 45.33%, respectively. About 95.69% of thermotolerant *E. coli* isolates were non-susceptible to six different antibiotics using BMA. Nearly 37% of the isolates were found to be multidrug-resistant (MDR) with most isolates resistant to ampicillin (81.72%). Furthermore, the occurrence of ESBL genes was significantly correlated with *tetA* genes ($P = 0.013$). To date, this study is the first to report on the presence of MDR and thermotolerant *E. coli* in Laguna Lake, Philippines.

**Key words:** lake water quality, multidrug resistance, Philippines, surface water, thermotolerant *E. coli*

## HIGHLIGHTS

- First report of the ecological presence of thermotolerant and multidrug-resistant *Escherichia coli* (37%) in Laguna Lake.
- Most (75.70%) isolates harbored at least one of the targeted antibiotic resistance genes (*strA*, *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$, and *tetA*).
- First report of the occurrence of aminoglycoside resistant gene (*strA*) and tetracycline efflux gene (*tetA*) in waterborne *E. coli* isolated in the Philippines.

## GRAPHICAL ABSTRACT




## INTRODUCTION

In recent decades, the prevalent and inappropriate uses of antibiotics in clinical and agricultural practices have led to the accelerated occurrence of resistance to antibiotic compounds (Osińska *et al.* 2017; Amarasiri *et al.* 2020). Discharges from wastewater treatment plants, hospital, and farming practices have subsequently reached the environment (Yang *et al.* 2018). The aquatic environments became catch basins of antibiotic compounds, antibiotic-resistant bacteria (ARB), and antibiotic resistance genes (ARGs) (Osińska *et al.* 2017; Amarasiri *et al.* 2020). For several years, the prevalence of ARB and ARGs in marine water (Cuadrat *et al.* 2020), watershed (Cho *et al.* 2019), river (Harnisz *et al.* 2020; Yang *et al.* 2020), lakes (Liang *et al.* 2020; Yang *et al.* 2020), groundwater (Szekeres *et al.* 2018), and even in tap water (Yang *et al.* 2020) have been widely documented. This occurrence of ARB and ARGs in the aquatic environment has been associated with the rapid spread of antibiotic-resistant pathogens in animals and humans (Al Salah *et al.* 2019; Amarasiri *et al.* 2020). This phenomenon is a growing health concern that might expedite the creation of multidrug-resistant (MDR) microorganisms that might be challenging to eliminate (Amarasiri *et al.* 2020). Also, negative health outcomes, mortality, and high hospital cost are some consequences of infections with ARB (Thorpe *et al.* 2018; Dadgostar 2019).

Recently, surveillances have focused on lakes, since standing or slow-flowing bodies of water are highly probable to retain ARB and ARGs (Czekalski *et al.* 2015; Yang *et al.* 2018). In the Philippines, increasing rates of resistance were observed on laboratory-based surveillances (Argimón *et al.* 2020). However, studies on ARB and ARGs in lakes in the Philippines are limited. Laguna Lake (Laguna de Bay) is the largest inland freshwater lake in the Philippines with a surface area of about 900 $km^2$ (Sta. Ana & Espino 2020). The lake is a significant zone for aquaculture, fisheries, industry, recreation and water source for irrigation and domestic uses (Fajardo & Ocampo 2018; Sta. Ana & Espino 2020). Sulfonamides and sulfamethoxazole resistance genes (*sul 1*, *sul 2*, and *sul 3*) were detected in selected sites in the lake in 2009 (Suzuki *et al.* 2013). Also, *Vibrio* and *Salmonella* spp. with phenotypic resistance to six antibiotics were detected in one of the river catchments of Laguna Lake (Ntabugi *et al.* 2020). However, little is known about the presence of antibiotic-resistant *Escherichia coli* in this lake.

*E. coli* is a clinically important Gram-negative bacterium commonly infecting both humans and animals. Some *E. coli* strains are harmless commensal of the gut, while some serotypes are pathogenic causing illness which includes diarrhea, urinary tract infections, respiratory illnesses, and sepsis, to name a few (Odonkor & Addo 2018; Swedan & Alrub 2019). It is a useful indicator species for microbial quality assessment, and its occurrence in the environment is highly associated with fecal

contamination from warm-blooded animals (Odonkor & Addo 2018). It is recognized as a suitable indicator for antibiotic resistance (Liu *et al.* 2018; Odonkor & Addo 2018). It is documented to carry various ARGs (Poirel *et al.* 2018; Haberecht *et al.* 2019) and is identified with an increasing rate of resistance to antibiotics which include aminoglycosides, fluoroquinolone, and $\beta$-lactam antibiotics (De Oliveira *et al.* 2020). In addition, *E. coli* strains with co-resistance to extended-spectrum $\beta$-lactamase (ESBL), aminoglycosides, and tetracycline were documented in water sources (Tacão *et al.* 2014). In this study, the presence of antibiotic-resistant *E. coli* in Laguna Lake was investigated. This study also aimed to detect the presence of antibiotic genetic determinants for ESBL ($bla_{\text{CTX-M}}$, $bla_{\text{SHV}}$, and $bla_{\text{TEM}}$), aminoglycoside (*strA*), and tetracycline (*tetA*) sampled from the surface water of Laguna Lake. Also, the co-occurrence of the selected genes was investigated.

## MATERIALS AND METHODS

### Sample collection

Laguna Lake as an important water source and catch basin of various rivers is consistently monitored by the Laguna Lake Development Authority (LLDA). There are 15 sampling lake stations established by the LLDA. In this study, water samples were collected from selected three sampling locations (Stations 2, 5, and 8). The stations were selected with the highest number of total coliforms based on the assessment of data acquired from the LLDA.

Station 2 is located at the east side of the lake; Station 5 is the sampling site nearest to Metro Manila; and Station 8 is on the South Bay (Figure 1). Water collection was done from October 2019 to January 2020 and March 2020. Approximately, 1 L of water samples were collected into sterile bottles and were immediately placed on ice, transported, and processed in the laboratory within 24–48 h.

### Bacterial isolation

Isolation of thermotolerant *E. coli* was conducted using the membrane filtration method by the US EPA as previously described (Labrador *et al.* 2020). Approximately, 1 L of water samples were filtered through a filter membrane (47 mm filter diameter, 0.45 μm pore size; Pall Corp., USA) using a vacuum pump. The membrane filters were incubated on the modified membrane-thermotolerant agar (mTEC; BD Difco, USA) at 35 °C for 2 h and then incubated to 42 °C for 18–24 h. Presumptive isolates characterized by blue to violet colonies were transferred to eosin methylene blue agar (EMBA; BD BBL, USA) plates for confirmation. After incubation, green metallic sheen colonies were selected and grown overnight on


**Figure 1** | Map showing locations of three sampling sites in Laguna Lake, Philippines. The inset shows the location of Laguna Lake in the Philippines.

trypticase soy broth (TSB; BD BBL, USA) at 35 °C for 18–24 h for further analysis. Separate tubes were also prepared in which glycerol (20%) was added after 18–24 h incubation for storage.

### Genomic DNA extraction and molecular identification

To extract DNA, a simple boil-lysis method was employed (Garcia *et al.* 2015). Isolates were grown on TSB for 18–24 h and were concentrated through centrifugation at 10,000 × g for 10 min. The resulting pellet was washed in 1 mL distilled water through centrifugation at 10,000 × g for 10 min, which was then suspended in 100 µL sterile water and heated at 100 °C for 15 min. Supernatant was collected in sterile tubes and kept for further analysis. Presumptive *E. coli* isolates were confirmed genotypically using *uidA* gene primers, ECN1254F and ECN1328R (Frahm & Obst 2003), following PCR cycling conditions previously described (Table 1). PCR mixture consisted of 1× GoTaq® Green Mastermix (Promega, USA), 0.50 µM of forward and reverse primers, 1 µL of DNA template, and appropriate nuclease-free water for a total volume of 12 µL. A no template mix and *E. coli* ATCC 25922 DNA were used as negative and positive controls, respectively.

### Detection of resistance genes

The presence of ARGs for aminoglycosides (*strA*) and tetracycline (*tetA*) was detected using singleplex PCR, while a multiplex PCR for *β*-lactams ($bla_{TEM}$, $bla_{CTX\text{-}M}$, and $bla_{SHV}$) was performed in confirmed 214 thermotolerant *E. coli* isolates. Amplification was carried out using 2 µL of DNA, 1× GoTaq® Green Mastermix (Promega, USA), 1 µM of forward and reverse primers, and nuclease-free water following the PCR conditions described in Table 1. Appropriate controls were used for each run. For *strA*, $bla_{CTX\text{-}M}$, and *tetA* genes, the positive controls were the isolates in this study, which were sequenced and confirmed with the deposited sequences in GenBank using the Basic Local Alignment Search Tool (BLAST).

After amplification, 3 µL of generated amplicons were separated on 2% agarose gel stained with SYBR® safe DNA gel stain (Invitrogen, USA) for 30 min at 280 V in Tris-Acetate-EDTA (TAE) buffer and visualized via the gel documentation system

**Table 1** | Primers and cycling conditions for detection of ARGs

| Gene | Antibiotic class | Primers (5′-3′) | PCR condition | Amplicon size (bp) | Positive control |
|---|---|---|---|---|---|
| ECN | – | GCAAGGTGCACGGGAATATT<br>CAGGTGATCGGACGCGT | 2 min at 98 °C, 35 cycles of 30 s at 95 °C, annealing for 1 min at 63 °C, elongation for 1 min at 72 °C, and extension for 5 min at 72 °C[a] | 75 | *E. coli* (ATCC EC25922) |
| *strA* | Aminoglycosides | TCAATCCCGACTTCTTACCG<br>CACCATGGCAAACAACCATA | 5 min at 95 °C, 35 cycles of 30 s at 95 °C, annealing for 1 min at 54 °C, elongation for 1 min at 72 °C, and extension for 5 min at 72 °C[b] | 126 | P15 |
| $bla_{CTX\text{-}M}$ | *β*-lactams | ATGTGCAGYACCAGTAARGTKATGGC<br>TGGGTRAARTARGTSACCAGAAYCAGC | 3 min at 95 °C, 29 cycles of 30 s at 95 °C, annealing for 30 s at 60 °C, elongation for 1 min at 72 °C, and extension for 10 min at 72 °C[c] | 593 | *Salmonella* sp. |
| $bla_{TEM}$ | | TCGCCGCATACACTATTCTCAGAATGA<br>ACGCTCACCGGCTCCAGATTTAT | | 445 | |
| $bla_{SHV}$ | | ATGCGTTATATTCGCCTGTG<br>TGCTTTGTTATTCGGGCCAA | | 745 | *K. pneumoniae* (ATCC 700603) |
| *tetA* | Tetracycline | GCTACATCCTGCTTGCCTTC<br>CATAGATCGCCGTGAAGAGG | 5 min at 94 °C, 35 cycles of 1 min at 94 °C, annealing for 1 min at 57 °C, elongation for 1 min at 72 °C, and extension for 7 min at 72 °C[d] | 210 | J596 (this study) |

[a]Labrador *et al.* (2020).
[b]Di Cesare *et al.* (2015).
[c]Monstein *et al.* (2007).
[d]Ng *et al.* (2001).

(Bio-Print ST4, Vilber Lourmat, UK). DNA ladders with sizes of 1 kb (KAPA Universal Ladder, Biosystem), 50, and 100 bp (Hyperladder™ Bioline) were used to estimate the molecular weights of the amplicons.

### Antimicrobial susceptibility test

Phenotypic antibiotic resistance was determined by broth microdilution assay (BMA) as previously described (Stachowiak *et al.* 2010; Balouiri *et al.* 2016). Ninety-three selected isolates with the identified presence of *β*-lactams or tetracycline resistance genes were tested to six antibiotics covering four different antimicrobial classes: kanamycin (32 μg/mL) and streptomycin for aminoglycosides; ampicillin (10 μg/mL) for *β*-lactams; kanamycin (32 μg/mL) and streptomycin (20 μg/mL) for aminoglycosides, imipenem (10 μg/mL) for carbapenem; ciprofloxacin (5 μg/mL) for quinolones and tetracycline (30 μg/mL).

Isolates in EMBA were streaked to trypticase soy agar (TSA; BBL, USA) and cultured for 18–24 h at 35 °C. The cultures were adjusted to 0.5 McFarland turbidity standard, and approximately 200 μL of cultures were inoculated to a 96-well microtiter plate to make a master plate. Approximately, 50 μL of cultures from the master plates were transferred to separate microtiter plates containing 50 μL TSB supplemented with 50 μL individual antibiotics. Resistance was defined as growth and sensitive as no growth using observable turbidity as measurement. *E. coli* ATCC 25922, drug, and medium (TSB) only containing wells were also included as controls.

### Statistical analysis

Frequency and graphs were generated using Microsoft Excel. Fisher's exact test was performed using SPSS v. 26 (SPSS Inc., Chicago, IL, USA) to establish an association among the tested antimicrobial-resistant genes. *P*-values of $\leq 0.05$ were considered significant.

## RESULTS AND DISCUSSION

The aquatic environment is recognized as reservoir of ARB and ARGs and is considered an important element for the transmission of antibiotic resistance (Osińska *et al.* 2017; Cho *et al.* 2019). As mentioned earlier, the occurrence of ARB and ARGs in the water system has been documented globally. Surveillance of ARB and ARGs is one of the efforts established in understanding the rise of resistance to antibiotic agents (Argimón *et al.* 2020). In this study, Laguna Lake was assessed for the occurrence of ARB and ARGs through the detection of thermotolerant *E. coli* isolates.

Two hundred and fourteen molecularly confirmed *E. coli* were isolated in three sampling stations. One hundred sixty-one (75.70%) were found to contain at least one of the target ARGs except for $bla_{\text{CTX-M}}$ (Table 2). Collectively, the ESBL $bla_{\text{TEM}}$ gene was dominantly observed among the five selected genes. The predominance of $bla_{\text{TEM}}$ was also observed in irrigation waters in the Philippines, where about 48% of isolates were positive for the gene (Vital *et al.* 2018). Also, $bla_{\text{TEM}}$ was more frequently observed in clinical isolates in Filipinos (Cruz & Hedreyda 2017).

Among the ARB, the spread of ESBL-producing *Enterobacteriaceae* is identified as a primary concern of the World Health Organization (WHO) in 2015 (Ng & Gin 2019). From the ESBL-producing bacteria, the temoneira (TEM) *β*-lactamases are produced due to mutations in TEM, which are encoded by $bla_{\text{TEM}}$ genes (Bailey *et al.* 2011; Baniga *et al.* 2020). TEM types are capable of deactivating classes of penicillin drugs such as ampicillin (Bailey *et al.* 2011). Recent reports of TEM harboring *E. coli* isolates sampled from water sources (Osińska *et al.* 2017; Baniga *et al.* 2020) were consistent with the findings in this

**Table 2** | Occurrence of ARGs in water samples collected in three sampling sites

| | | Sampling stations | | | |
|---|---|---|---|---|---|
| **Antibiotic class** | **Target gene** | **Station 2** ***n*** **= 77** | **Station 5** ***n*** **= 78** | **Station 8** ***n*** **= 59** | **Total (%)** ***n*** **= 214** |
| Aminoglycosides | *strA* | 48.05 | 13.43 | 24.53 | 29.91 |
| *β*-lactams | $bla_{\text{CTX-M}}$ | 0.00 | 0.00 | 0.00 | 0.00 |
| | $bla_{\text{SHV}}$ | 12.99 | 5.97 | 3.77 | 7.88 |
| | $bla_{\text{TEM}}$ | 51.95 | 71.64 | 20.75 | 47.66 |
| Tetracycline | *tetA* | 55.84 | 55.22 | 22.64 | 45.33 |

study. However, our findings seem to disagree with the recent report of isolates sampled from selected hospital sewers and rivers in the Philippines, wherein *E. coli* isolates were found to carry $bla_{\text{CTX-M}}$ genes (Suzuki *et al.* 2020). The variations in results of the prevalent ESBL types might be attributed to differences in sampling period and geographical locations (Vital *et al.* 2018).

Aminoglycosides are antibiotics that are frequently used in combination with β-lactams for the treatment of Gram-negative bacterial infections (Bodendoerfer *et al.* 2020). One mechanism of resistance of aminoglycosides involves the formation of enzymes, which modify the antibiotic drug at various sites (Bodendoerfer *et al.* 2020; Schaenzer & Wright 2020). The *strA* gene is one of the resistance genes that encode aminoglycoside-modifying enzyme, which confers resistance to streptomycin (Frye & Jackson 2013; Poirel *et al.* 2018). In this study, 29.91% of *E. coli* isolates were positive for *strA* genes (Table 2). To date, this is the first report of detection of *strA* gene in Philippine waters. Similar findings of the occurrence of *strA* gene were reported in waterborne (Gomi *et al.* 2017; Singh *et al.* 2021) and foodborne *E. coli* isolates (Zhang *et al.* 2014; Poirel *et al.* 2018) in several countries.

The presence of *tetA* gene against tetracycline was also detected in this study. It was shown that almost half of *E. coli* isolates have *tetA* gene (Table 2). A study on fruits, spices, and vegetables sourced from selected markets in the Philippines has found high prevalence of the *tetA* gene (30%) in *E. coli* isolates (Vital *et al.* 2017). To our knowledge, this present study is the first detection of *tetA* from Philippine waters. The *tetA* gene is commonly observed in aquatic environment (Cho *et al.* 2019; Liang *et al.* 2020), supporting the result of our study. Tetracyclines are generally applied on animals, and it was hypothesized that their presence in the aquatic system might indicate the contamination of ARGs from animal farming (Czekalski *et al.* 2015).

The coexistence of different ARGs within the same isolate has been established (Poirel *et al.* 2018; Bodendoerfer *et al.* 2020; Liang *et al.* 2020). The establishment of co-occurrence is significant for guiding combination therapy to limit resistance in antibiotic compounds (Bodendoerfer *et al.* 2020). In this study, significant association was observed between ESBL and *tet*A gene (Table 3). Similar significant co-resistance was observed between *tetA* + and ESBL+ isolates in waterborne *E. coli* from other countries (Tacão *et al.* 2014). In contrast, our findings show no significant relationship of *strA* to *tetA* + and ESBL+ isolates. Further study is warranted, since significant co-occurrence was observed with aminoglycosides genes *aph(3′)-Ia* or *aac(3)-IId* and $bla_{\text{TEM-1}}$ in clinical isolates in the study of Bodendoerfer *et al.* (2020).

In this present study, 93 ESBL+ and *tetA* + isolates were selected from the 214 *E. coli* isolates for phenotypic characterization. The majority (95.69%) of the isolates exhibited at least one phenotypic resistance to the six antibiotics tested encompassing four different antibiotic classes. Most isolates were resistant to ampicillin, a β-lactam antibiotic, followed by streptomycin and tetracycline (Table 4). On the other hand, few isolates were observed to be nonselective to iminepem and ciprofloxacin (Table 4).

Commonly, MDR bacteria are defined as having non-susceptibility to at least three or more antimicrobial categories (Osińska *et al.* 2017; van Spijk *et al.* 2019). Although the number of isolates was uneven in sampling locations, it is noteworthy that the presence of MDR was evident in all three sampling locations (Figure 2(a)–2(c)). Overall, 37% were MDR being non-susceptible to three or more antibiotics, and 39% were resistant to two antibiotics tested (Figure 2(d)). Similar observations were shown on *E. coli* strains from irrigation water collected in various areas of Metro Manila, Philippines. Paraoan *et al.* (2017) recorded 58.22% MDR isolates collected in Bulacan and 36.5% MDR isolates were documented from urban farms in different areas of Metro Manila (Vital *et al.* 2018). Furthermore, phenotypic assay has shown resistant *E. coli* isolates against β-lactams from isolates recovered from Lake Lanao, Philippines (Barosa *et al.* 2020). Taken together, although the pathogenicity of the sampled isolates in this study was not determined, our findings provided information on the occurrence of thermotolerant and MDR *E. coli* isolates in Laguna Lake.

**Table 3** | Fisher's exact test results showing association among the ARGs

| | ***bla*** | ***strA*** | ***tetA*** |
|---|---|---|---|
| *bla* | – | 0.299 | 0.013* |
| *strA* | 0.299 | – | 0.374 |
| *tetA* | 0.013* | 0.374 | – |

*Significant at $P \leq 0.05$ (two-tailed).

**Table 4** | Antibiotic resistant profile of isolated thermotolerant *E. coli* from Laguna Lake

| Antibiotic agent | Dosage (μg/mL) | Resistant isolates (%) $n = 93$ |
|---|---|---|
| Kanamycin | 32 | 18.28 |
| Streptomycin | 20 | 62.36 |
| Ampicillin | 10 | 81.72 |
| Iminepem | 10 | 18.28 |
| Ciprofloxacin | 5 | 11.82 |
| Tetracycline | 30 | 29.03 |

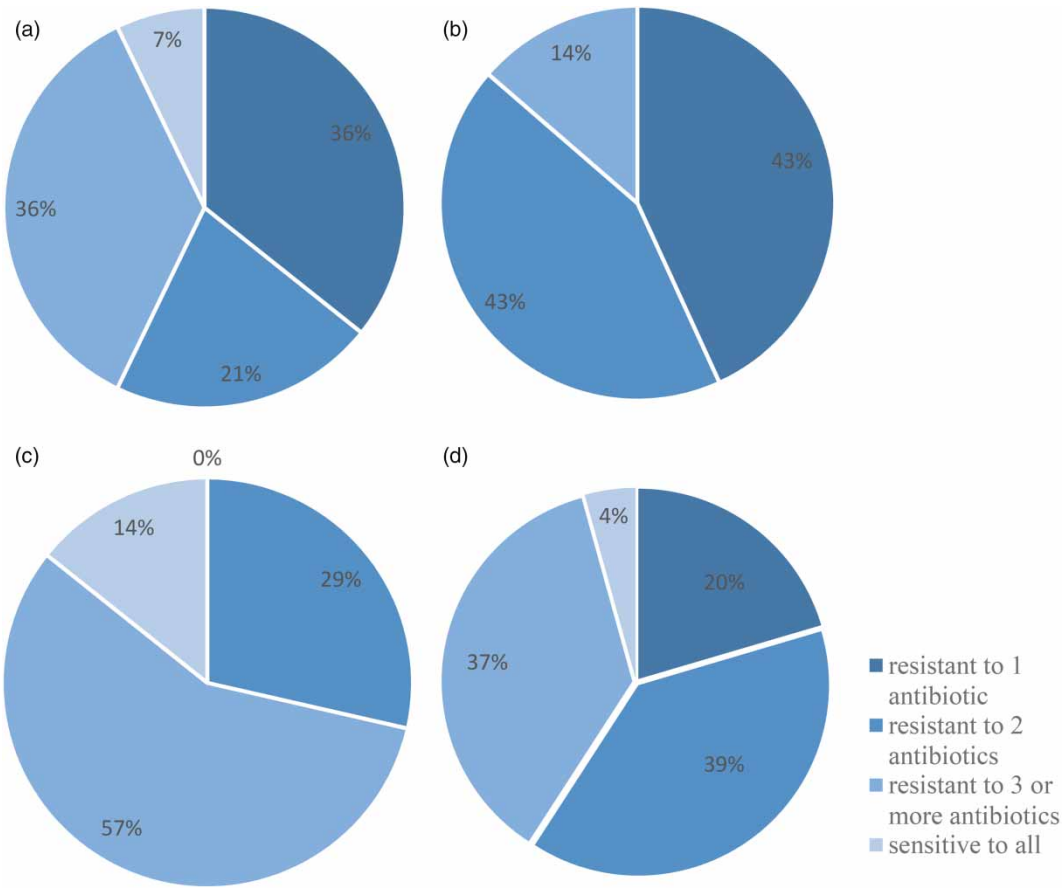



**Figure 2** | MDR patterns of *E. coli* isolates in (a) Station 2 ($n = 42$), (b) Station 5 ($n = 44$), (c) Station 8 ($n = 7$), and (d) all sampling points.

## CONCLUSION

This study isolated MDR *E. coli* in Laguna Lake. Isolates were found to be phenotypically resistant to four different antibiotic classes and were reservoir of four resistance genes (*strA*, $bla_{\mathrm{SHV}}$, $bla_{\mathrm{TEM}}$, and *tetA*) covering three different classes of antibiotic agents such as aminoglycosides, β-lactams, and tetracycline. The TEM carrying *E. coli* was highly observed among the isolates. Also, this is the first detection of *strA* + and *tetA*+ *E. coli* isolates in Philippine waters. Furthermore, significant association between ESBL+ and *tetA* + isolates was observed in this study. Taken together, these results are an addition to the growing evidence of the ecological presence of ARB and ARGs.

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## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

## DATA AVAILABILITY STATEMENT

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

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