Antibiotic resistance of esculin-hydrolyzing bacteria isolated from tilapia farms with different water sources in Pampanga, Philippines

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Abstract. The study aimed to assess the antibiotic resistance of esculin-hydrolyzing bacteria isolated from river-dependent and groundwater-dependent farms located in Pampanga, Philippines. Samples of water, sediments and fish were collected for the isolation of the target bacteria. Antibiotic susceptibility test was conducted using the Kirby-Bauer disc diffusion method. The result of the preliminary test indicated high rate of resistance among isolates from both farm types to ampicillin, rifampicin, vancomycin, linezolid, penicillin and erythromycin. Moreover, selected isolates from both farm types obtained a multi-antibiotic resistance index (MARI) of > 0.2, suggesting that these areas are at high risk of antibiotic contamination. Sequencing of selected multi-antibiotic resistant isolates revealed that *Klebsiella* spp. dominate in river-dependent farms while *Aeromonas* spp. are common in groundwater-dependent farms.
Key Words: groundwater-dependent, multi-antibiotic resistant, river-dependent, tilapia farms.

Introduction. Bacterial pathogens have long been studied as primary factor in the outbreaks of diseases in fish farming. Intensification of production may result to water quality degradation, which in turn favors bacterial proliferation and accumulation causing mortalities, threatening sustainable production (Haenen et al 2023). Without any intervention, these factors can promote bacterial infection, which could possibly become an important vector for environmental contamination and human foodborne illnesses (Thaotumpitak et al 2022). Among the major farmed fish commonly studied for bacterial infection is Nile tilapia (*Oreochromis niloticus*). Although the fish demonstrated relative resistance to many bacterial attack, recent investigations showed the susceptibility of farmed species to various infectious diseases (Debnath et al 2023).

Traditionally, antimicrobial methods are implemented to control the occurrence and proliferation of pathogens in fish farms. The application of antibiotics is widely used to treat and control bacterial diseases; however, the amount of antibiotics used in aquaculture is difficult to quantify (Romero et al 2012). Of the 67 types of antibiotic compounds used by major aquaculture producers worldwide, oxytetracycline, sulphadiazine and florfenicol are commonly used (Lulijwa et al 2020). Antibiotic chemotherapy is usually made by incorporating the compounds to the diet of fish (Okocha et al 2018).

As mentioned, most of the antibiotics used in aquaculture are effective against Gram-negative bacteria (Bondad-Reantaso et al 2023). However, indiscriminate use exerts selective pressure driving antimicrobial resistance (Schar et al 2021). One of the most studied topics in the area of pathogenic bacteria is the concept of antimicrobial resistance (AMR). This natural phenomenon is considered a global health threat involving pathogenic bacteria and such risk related to the use of antimicrobial agents among food production sectors may include the spread of resistant pathogens and resistant genes via food chain, which can cause zoonotic diseases (Gazal et al 2020). Hence, this problem should not be overlooked and thorough investigation is indispensable (Kam et al 2022).

Based on the report of Roh et al (2016), the resistance of bacteria to various antibiotics increased significantly in fish farms. In theories, the emergence of resistant strains could eliminate susceptible bacteria from the heterogeneous populations, of which resistant ones considerably proliferate. It has been reported that 90% of bacteria from the aquatic environment showed resistance to at least one type of antibiotic and about 20% are multi-antibiotic resistant (Pepi & Focardi 2021). Several factors have been recognized to influence the acquisition of resistance (Watts et al 2017; Pepi & Focardi 2021; Yuan et al 2023). Prolonged exposure to various antibiotics aids the development of resistance in aquaculture systems. Moreover, climate change worsened and magnified the problem. Studies have linked the occurrence of antibiotic resistance in fish farming to the provision of artificial diets incorporated with antibiotics as well as direct application in pond water and inappropriate disposal of wastes from animal husbandry, domestic, hospital and industrial areas that may accumulate in water and sediment (Agoba et al 2011; Adinortey et al 2020; Yuan et al 2023).

Aquaculture has been implicated as major driver in broad dissemination of antibiotic resistance among pathogens affecting both humans and animals (Dewi et al 2022). Nowadays, aquaculture has been regarded as an important source, hotspot and reservoir (Wang et al 2021). High incidence of antibiotic resistant bacteria can be found in integrated and manure-fertilized ponds (Petersen et al 2002; Neela et al 2015; Fakorede et al 2020). Application of manures of animal origin, particularly those sources that received medications became source of further environmental diffusion of antibiotic resistance gene (Wamala et al 2018; Ferri et al 2022). Similarly, incidence rate is high in farms frequently administered with these compounds (Miranda et al 2018). However, resistant bacteria were also detected in fish farms with no history of antibiotic application, suggesting the possibility of cross-contamination from other systems (Hossain et al 2021). From this viewpoint, water can be considered as a primary medium of transmission.

The province of Pampanga is well-known for its huge contribution of farm-produced Nile tilapia in the Philippines. As mentioned, its farming system is heavily dependent on river tributaries and canals as primary sources of water; however, there are some farms that are dependents on deep-well or groundwater sources as these areas are elevated and relatively distant from the mighty Pampanga River that supplies the water requirement for agriculture and fisheries (Reyes 2018; Reyes & Reyes 2019). Considering the paucity of information regarding antibiotic resistance in tilapia aquaculture, this undertaking was done to investigate the response of esculin-hydrolyzing bacteria from Nile tilapia farms with different water sources against a pool of antibiotics commonly used in human medicine and animal production.

Material and Method

Bacterial isolation. Samples of water, sediment and fish were collected in triplicates from pre-identified river-dependent and groundwater-dependent farms in Minalin and Magalang, Pampanga from October to November 2023. The samples were transported to the laboratory within 6 hours using Styrofoam box with ice. One (1) gram of water, sediment and homogenous tissues from fish (skin, muscle, gut, liver, and brain) was serially diluted ($10^{-1} - 10^{-6}$) in sterile physiological saline at 10% concentration. One hundred (100) µL aliquots from serially diluted samples were spread to bile-esculin agar (Himedia Laboratories Pvt. Ltd., Mumbai, Maharashtra, India) plates and incubated for 18-24 hours at 37°C (Adeniji et al 2021). Major colony showing dark coloration emerged in each agar plate was selected and subjected to series of purification. The purified isolate was used for antibiotic susceptibility test.

Antibiotic susceptibility testing. The susceptibility of the isolates was tested on 11 types of antibiotics, which include ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), ciprofloxacin (5 µg), linezolid (30 µg), nitrofurantoin (300 µg), norfloxacin (10 µg), penicillin (10 µg), rifampicin (5 µg), tetracycline (30 µg), and vancomycin (30 µg). Majority are being used in human medicine and animal production.

The test involved Kirby-Bauer disc diffusion method in Muller-Hinton Agar (MHA). Two to three colonies observed in the isolates were randomly selected and transferred using sterile inoculating loop to test tube containing 5 mL of sterile physiological saline (0.9%) and vortexed to produce a suspension matching to 0.5 McFarland standard (Adeniji et al 2021). Using a sterile single channel micropipette, 100 µL of the suspension was transferred to the agar plate (150 mm Petri dish), uniformly spread using a sterile L-rod and allowed to dry for 5 minutes. After drying, six (6) commercial antibiotic discs (including the control) were impregnated in the agar plates (90 mm diameter) at an equidistant spacing using disc dispenser and sterile forceps. The plates were subjected to 24-hour incubation in an inverted position at 37°C (Igbinosa 2016).

Assessment of antibiotic resistance/susceptibility. The diameter of the zone of inhibition around each disc was measured using a digital Vernier caliper and recorded to nearest millimeter (mm). The measurements were compared to the standard interpretative chart provided by the Clinical and Laboratory Standards Institute (CLSI) (2021) for enterobacterales and enterococci to determine the susceptibility (S), intermediate (I) and resistance (R) of isolates against the pool of antibiotics. Isolates exhibiting antibiotic resistance were identified.

Determining multi-antibiotic resistance. After a preliminary susceptibility test, 10 isolates (regardless of sources) from each farm type were selected based on the number of antibiotics they are resistant with. In cases where many isolates showed resistant to the same number of antibiotics, random selection was done. The selected isolates were again subjected to susceptibility testing to verify the previous result and to determine whether there is multi-drug resistant or none. Determining the multi-antibiotic resistant (MAR) isolate was based on the description of Magioarakos et al (2012), in which a minimum of three antibiotic classes will be used as reference point. The antibiotic resistant isolates were interpreted using the formula for Multiple Antibiotic Resistance Index (MARI) (Krumperman 1983):

$$MARI = \frac{Number\ of\ antibiotics\ to\ which\ resistance\ occurred}{Number\ of\ antibiotics\ tested}$$

This index is considered an efficient method of tracking antibiotic resistant organisms (Sandhu et al 2016). A value of 0.2 was used as reference point in order to differentiate low-risk and high-risk farms (Davis & Brown 2016). A value of more than 0.2 indicates that isolates are from high-risk sources (Kathleen et al 2016).

Bacterial identification. Bacterial identification was done by submitting pure isolated culture to a private sequencing company. Inclusive in the services are DNA extraction, PCR amplification and purification, bidirectional sequencing and contig assembly. Based on their protocol, extraction involved the use of 20 µL distilled water (DW), 20 µL ThermoFisher prepman and the sample colony (spin down to 13000 rpm for 1 min). After extraction, only 1.5 µL of the supernatant was used for PCR amplification. Amplification was carried out using 16S 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') universal primers. The PCR mixtures containing the bacterial DNA (20 µL) were amplified using DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD). The process stared with the activation of Taq polymerase (MBAE-110 Axen H Taq PCR Master Mix, 2x) at 95°C for 5 mins, followed by denaturation at 95°C for 30 sec, annealing process 35 cycles at 55°C for 30 sec and extension at 72°C for 1 min, with final extension at 72°C for 7 mins. The sequences of the PCR products were subjected to cleaning and analyzed with nucleotide Basic Alignment Search Tool (BLASTn) provided by the National Center of Biology Information (NCBI).

Results. Based on the result of preliminary test (Tables 1-3), isolates of esculin-hydrolyzing bacteria are mostly resistant towards six (6) types of antibiotics. These include ampicillin, erythromycin, linezolid, penicillin, rifampicin and vancomycin.

Comparing the response in accordance with water sources (Table 2), highest resistance rate to rifampicin and vancomycin (86.67%) was observed in river-dependent while resistance to linezolid and rifampicin (86.67%) was recorded in groundwater-dependent farms. Meanwhile, it was found that isolates from the water of river-dependent farms are susceptible to other kinds of antibiotics such as chloramphenicol, ciprofloxacin, nitrofurantoin, norfloxacin, and tetracycline. As to groundwater-dependent farms, some are resistant to chloramphenicol (6.67%) and nitrofurantoin (6.67%) but all isolates showed susceptibility to ciprofloxacin, norfloxacin and tetracycline.

Table 1

Resistance rate of isolates from water sources towards antibiotics

Antibiotics	Resistance rate of isolates to antibiotics (%)	
	River-dependent farms	Groundwater-dependent farms
Ampicillin	73.33	80.00
Chloramphenicol	0.00	6.67
Erythromycin	53.33	40.00
Ciprofloxacin	0.00	0.00
Linezolid	73.33	86.67
Nitrofurantoin	0.00	6.67
Norfloxacin	0.00	0.00
Penicillin	80.00	53.33
Rifampicin	86.67	86.67
Tetracycline	0.00	0.00
Vancomycin	86.67	80.00

With regard to sediment sources (Table 2), majority of the isolates from river-dependent farms showed higher resistance to linezolid (80.00%), rifampicin (80.00%) and vancomycin (80.00%) while the highest resistance in groundwater-dependent farms was towards ampicillin (73.33%). As also indicated, isolates from river-dependent farms showed higher resistance against some antibiotics to include erythromycin, penicillin, rifampicin and vancomycin compared to the isolated from the sediments of groundwater-dependent farms. However, antibiotics such as chloramphenicol, nitrofurantoin, and norfloxacin effectively suppressed the isolates from both farm types.

Table 2

Resistance rate of isolates from sediment sources to antibiotics

Antibiotics	Resistance rate of isolates to antibiotics (%)	
	River-dependent farms	Groundwater-dependent farms
Ampicillin	73.33	73.33
Chloramphenicol	0.00	0.00
Erythromycin	40.00	20.00
Ciprofloxacin	0.00	0.00
Linezolid	80.00	46.67
Nitrofurantoin	0.00	0.00
Norfloxacin	0.00	0.00
Penicillin	66.67	40.00
Rifampicin	80.00	26.67
Tetracycline	6.67	6.67
Vancomycin	80.00	40.00

In terms of bacterial isolates from fish (Table 3), highest resistance was towards ampicillin (100.00%) and rifampicin (100.00%) for river-dependent and only towards vancomycin (93.33%) for groundwater-dependent farms. Meanwhile, susceptibility was observed towards chloramphenicol, ciprofloxacin, norfloxacin and tetracycline. The same

response was found on isolates from groundwater farms except for tetracycline (6.67%) as few showed resistance. Looking deeper at the details, higher rate of resistance was found in river-dependent isolates than those from groundwater farms in general. However, higher number of antibiotics to which the isolates are resistance with was recorded in groundwater-dependent farms.

Table 3

Resistance rate of isolates from fish sources towards antibiotics

Antibiotics	Resistance rate of isolates to antibiotics (%)	
	River-dependent farms	Groundwater-dependent farms
Ampicillin	100.00	80.00
Chloramphenicol	0.00	0.00
Erythromycin	33.33	40.00
Ciprofloxacin	0.00	0.00
Linezolid	80.00	60.00
Nitrofurantoin	0.00	0.00
Norfloxacin	0.00	0.00
Penicillin	93.33	73.33
Rifampicin	100.00	86.67
Tetracycline	0.00	6.67
Vancomycin	93.33	93.33

Based on pooled data (Table 4), isolates from river-dependent farms were mostly resistant to six (6) types of antibiotics including rifampicin (88.89%), vancomycin (86.67%) ampicillin (82.22%), penicillin (80.00%), linezolid (77.78%) and erythromycin (42.22%), with few isolates that were resistant to tetracycline (2.22%). At the same time, none of these isolates were resistant to chloramphenicol, nitrofurantoin and norfloxacin. Meanwhile, there were higher number of antibiotics to which the isolates were resistance in groundwater-dependent farms. However, lower rate of resistance was recorded in terms of ampicillin (77.78%), vancomycin (71.11%), rifampicin (66.67%), linezolid (64.44%) and erythromycin (33.33%). Isolates from these farms showed only susceptibility towards ciprofloxacin and norfloxacin.

Table 4

Resistance rate of pooled isolates towards antibiotics

Antibiotics	Resistance rate of isolates to antibiotics (%)	
	River-dependent farms	Groundwater-dependent farms
Ampicillin	82.22	77.78
Chloramphenicol	0.00	2.22
Erythromycin	42.22	33.33
Ciprofloxacin	0.00	0.00
Linezolid	77.78	64.44
Nitrofurantoin	0.00	2.22
Norfloxacin	0.00	0.00
Penicillin	80.00	55.56
Rifampicin	88.89	66.67
Tetracycline	2.22	4.44
Vancomycin	86.67	71.11

Table 5 and 6 indicate the computed MARI of selected isolates from river-dependent farms and groundwater-dependent farms, respectively. Most of the representative isolates in river-dependent farms were from water samples while in groundwater-dependent farms, mostly were from fish samples. This was a result of random selection. Nevertheless, the result of further antibiotic susceptibility testing revealed that all selected

isolates from both farms showed MARI of > 0.2, indicating that the obtained isolates of esculin-hydrolyzing bacteria are from high-risk areas contaminated with antibiotics.

Table 5

Multiple antibiotic resistance index of selected isolates from river-dependent farms with the highest number of antibiotics to which they are resistant

Isolate	No. of antibiotics	MARI	Remarks
A1 (P1W1)	5.33±1.15	0.48±0.10	> 0.2 (high-risk)
A2 (P1S2)	6.00±0.00	0.55±0.00	> 0.2 (high-risk)
A3 (P2W2)	6.67±0.58	0.61±0.05	> 0.2 (high-risk)
A4 (P2F1)	6.67±0.58	0.61±0.05	> 0.2 (high-risk)
A5 (P3W2)	6.00±0.00	0.55±0.00	> 0.2 (high-risk)
A6 (P4W2)	6.00±0.00	0.55±0.00	> 0.2 (high-risk)
A7 (P4F1)	6.33±0.58	0.58±0.05	> 0.2 (high-risk)
8A (P4F2)	6.00±0.00	0.55±0.00	> 0.2 (high-risk)
A9 (P5S3)	6.00±0.00	0.55±0.00	> 0.2 (high-risk)
A10 (P2W3)	6.33±0.58	0.58±0.05	> 0.2 (high-risk)

Table 6

Multiple antibiotic resistance index of selected isolates from groundwater-dependent farms with the highest number of antibiotics to which they are resistant

Isolate	No. of antibiotics	MARI	Remarks
B1 (P1W1)	6.00±0.00	0.55±0.00	> 0.2 (high-risk)
B2 (P1F1)	6.00±0.00	0.55±0.00	> 0.2 (high-risk)
B3 (P1F2)	6.67±0.58	0.61±0.05	> 0.2 (high-risk)
B4 (P2F3)	5.00±0.00	0.45±0.00	> 0.2 (high-risk)
B5 (P3S1)	5.67±0.58	0.52±0.05	> 0.2 (high-risk)
B6 (P4W1)	7.67±0.58	0.70±0.05	> 0.2 (high-risk)
B7 (P5W2)	7.00±0.00	0.64±0.00	> 0.2 (high-risk)
B8 (P5F1)	7.00±1.73	0.64±0.16	> 0.2 (high-risk)
B9 (P5F2)	5.00±0.00	0.45±0.00	> 0.2 (high-risk)
B10 (P5F3)	4.33±0.58	0.39±0.05	> 0.2 (high-risk)

As shown in Table 7, the identified bacterial isolates belong to the genera of *Acinetobacter*, *Aeromonas*, *Enterobacter*, *Klebsiella*, *Kosakonia*, *Phytobacter*, and *Superficieibacter*. In river-dependent farms, most of the isolates are *Klebsiella* sp. (60.00%) while in groundwater-dependent farms, *Aeromonas* sp. (40.00%) dominate. *Acinetobacter*, *Enterobacter*, *Kosakonia*, and *Phytobacter* were not found in the selected isolates of river-dependent farms while *Superficieibacter* is not present in groundwater-dependent farms.

Table 7

Bacterial species identification of selected isolates from river-dependent and ground-water dependent farms after BLASTn analysis

| Species | Distribution (%) | |
	River-dependent farms	Groundwater-dependent farms
Acinetobacter sp.	0.00	10.00
Aeromonas sp.	30.00	40.00
Enterobacter sp.	0.00	20.00
Klebsiella sp.	60.00	10.00
Kosakonia sp.	0.00	10.00
Phytobacter sp.	0.00	10.00
Superficieibacter sp.	10.00	0.00

Discussion. Of the 11 types of antibiotics tested, the isolates showed high resistance rate towards ampicillin, rifampicin, vancomycin, linezolid, penicillin and erythromycin. The result suggests the contamination between farm types to these kinds of antibiotics.

Ampicillin is an extended spectrum of penicillin that is widely used against Gram-positive as well as Gram-negative microorganisms, specifically to manage and treat certain bacterial infections (Kaushik et al 2014; Peechakara et al 2023). In aquaculture, ampicillin is among the most commonly used β-lactams (Bondad-Reantaso et al 2023). However, because of widespread dependence on β-lactams resistance increases (Vasoo et al 2015), several bacterial organisms, especially the Gram-negative ones become resistant to β-lactams due to the production of wide range of β-lactamase enzyme (Karami et al 2008; Bush & Bradford 2016). This enzyme hydrolyzes the opening of β-lactam ring, rendering the antibiotics unable to target the penicillin-binding proteins (PBPs) (Mora-Ochomogo & Lohans 2021). Isolates of aquatic Gram-negative bacilli exhibit high resistance rate (76.29%) against ampicillin (Sivri et al 2012). Isolates of *Aeromonas* spp. from *O. niloticus* and *C. gariepinus* in Uganda were reported to have 100% resistant rate towards penicillin and ampicillin (Wamala et al 2018).

Rifampicin is another antibiotic frequently used in aquaculture; however, the effectiveness of this antibiotic and other types of rifamycins are now declining due to the occurrence of resistant bacterial strains (Bondad-Reantaso et al 2023). It has been described that resistance to rifampicin arises when the β-subunit is substituted by a single amino acid (Hughes & Brandis 2013). Resistance to rifampicin has been extensively investigated with *E. coli* (Rodriguez-Vedugo et al 2013), but resistance was also reported to some fish bacterial pathogens (Gufe et al 2022; Gao et al 2023).

Resistance to linezolid is uncommon and rarely encountered phenomenon among Gram-positive bacteria. Moreover, the antibiotic is ineffective against Gram-negative bacteria due to an efflux mechanism (Winston et al 2009). Emergence of linezolid-resistant bacteria is associated with the mutations in the genes of ribosomal proteins or acquisition of cfr gene (Kosecka-Strojek et al 2020; Lienen et al 2022). Isolates of enterococci from fish were found to have a resistant rate of 69.23% (Rana et al 2023).

Vancomycin, a glycopeptide, is commonly used as treatment for severe infections caused by multi-drug resistant Gram-positive cocci (Olufunmiso et al 2017; Muhlberg et al 2020). However, resistant strains are increasing nowadays. Vancomycin resistance among these bacteria is due to the alteration of peptidoglycan terminus, resulting the reduction of the antibiotic binding capacity (Kumar 2017). Similar to linezolid, vancomycin is generally ineffective to Gram-negative bacteria. This is attributed to its relatively large molecular size, rendering inability to penetrate the outer membrane barrier of the organisms (Fernandes et al 2017; Antonoplis et al 2019).

The resistance to erythromycin, a macrolide has been commonly linked to the modification of ribosomal binding site as a result of methylase synthesis (Mahfouz et al 2023). Other mechanisms include the elimination of the antibiotics by efflux system and the production of macrolide phosphorylases. In the study of Marijani (2022), enteric bacteria isolated from marine and freshwater fishes exhibited resistance to some kinds of antibiotics including erythromycin. A study on *E. coli* and *Salmonella* from farmed-raised red tilapia showed high resistance rate (Dewi et al 2022).

Meanwhile, susceptibility and low resistance rate were observed towards chloramphenicol, ciprofloxacin, nitrofurantoin, norfloxacin and tetracycline. The result suggests that these antibiotics are still effective in suppressing the growth and potential threat of esculin-hydrolyzing bacteria in the area. Chloramphenicol inhibits protein synthesis by binding to the 50S ribosomal subunits of the bacterium. Similarly, tetracycline disrupts protein synthesis but by binding to the 30S subunit of the ribosome, thereby preventing the association of aminoacyl-tRNA to the messenger RNA molecule (Chopra & Roberts 2001). Ciprofloxacin is an antibiotic in the fluoroquinolone class and it suppresses bacteria by disrupting their ability to create and repair DNA. This antibiotic inhibits most strains (> 90%) of Gram-negative aerobes and certain Gram-positive bacteria (Doble 2007). The bactericidal effect of nitrofurantoin is on the inhibition of carbohydrate synthesis by interfering the conversion of pyruvate to acetyl coenzyme A. Nitrofurantoin is bactericidal against *Enterobacter*, *Klebsiella*, *Enterococcus* and other

enteric bacteria; resistance to this antibiotic remains relatively rare (Squadrito & del Portal 2023). Lastly, the mechanism of norfloxacin to suppress bacteria is by inhibiting the A subunit of the important enzyme DNA gyrase (Goldstein 1987).

As revealed in this study, the isolates of multi-antibiotic resistant esculin-hydrolyzing bacteria from both farms belong to Gram-negative coliform category, of which considered as good indicator of fecal contamination. The high resistance to penicillin, ampicillin and erythromycin is in line with the report of Nchanji et al (2021) in the isolates of Gram-negative coliform bacteria from earthen tilapia ponds. It is important to point out that the incidence of resistance towards β-lactams and macrolides could be due to the frequency of their usage in animal and human medicine. Meanwhile, high resistance observed to linezolid and vancomycin can be attributed to the innate ability of Gram-negative isolates to resist the effect of these antibiotics. Previous studies also showed the resistance of some esculin-hydrolyzing bacteria to other antibiotics. Studied isolates of *Enterobacter* from fish ponds are resistant to various kinds of antibiotics to include ceftazidime, cefuroxime, cefixime and amoxicillin/clavulanic acid (Ayedun et al 2022). *Acinetobacter* was also identified in this study; however, it is not an esculin-positive one. Isolation suggests its ability to grow in the medium. It is commonly regarded as a major factor in transmitting antibiotic resistance gene. The species *A. baumanii* was reported to have extraordinary capacity to develop resistance towards multiple antibiotics (Dijkshoorn et al 2007). Similarly, the species *A. lwoffi* is resistant to a range of antibiotics and regarded as a causative agent for nosocomial infections (Dadar et al 2016). In the study of Kozinzka et al (2014), several *Acinetobacter* isolates from fish showed resistance to ampicillin, amoxicillin and cephalothin. *Aeromonas* could be one of the most studied pathogens in tilapia as it is typically associated with unwarranted diseases and mortality (Pakingking et al 2020). *Aeromonas* spp. are resistant to several types of antibiotics and can be spread to humans through consumption of fish or direct contact with fish environment (Sherif & Kassab 2023). *Aeromonas* strains are good indicator of antibiotic resistance development in fish farms (Patil et al 2016). As reviewed, isolates from freshwater fish farms are resistant to β-lactam and lincosamide antibiotics, but susceptible towards aminoglycosides, chloramphenicol and fluoroquinolones (Zdanowicz et al 2020). Recently, *A. hydrophila* isolated from tilapia grown in brackishwater ponds showed high resistance rate to ampicillin, amoxicillin and erythromycin (Pakingking et al 2020). Several reports indicated multi-antibiotic resistance of some esculin-hydrolyzing bacteria from fish and their environment (Sarter et al 2007; Adinortey et al 2020; Tsafack et al 2021; Preena et al 2022). For example, *Enterobacter* and *Klebsiella* spp. isolated from small-medium scale tilapia farms in Nigeria were found to have a MAR index of 0.5 to 0.9 (Fakorede et al 2020).

Even though farm owners/caretakers stated that they are not using antibiotics, there is high rate of resistance among isolates from water, sediment and fish against several types of antibiotics. This relatively high rate of resistance connotes the existence of antibiotic residues and contamination. Nearby rivers bring antibiotic compounds from domestic and hospital wastewaters in the farming systems, and ponds that are difficult for water exchange allow antibiotics to impose selective pressure on bacteria (Lin et al 2023). According to farm caretakers, they seldom change pond water because of tidal schedule and expensive cost of pumping underground water. This practice could increase the probability of occurrence of antibiotic resistant bacteria. Studies have also linked the occurrence of antibiotic resistant bacteria in fish farms to livestock and poultry production. The transmission via water could potentially facilitated through draining of wastewaters into natural water bodies such as lakes, rivers and swamps (Deng et al 2023). Farms participated in the current undertaking are located in areas where poultry or swine production is carried out. Although not studied, it is plausible that antibiotic residues, resistant bacteria, and resistant genes could enter the ponds from these areas through direct discharge of wastewaters to rivers or indirect transfer through run-offs. Moreover, it has been observed in this study that caretakers are keeping other animals such as chickens, ducks, pigs, dogs and goats roaming around the farm premises. These animals could also be potential factor for bacterial pollution (Deng et al 2023).

Conclusions. The study provides first-hand information regarding the presence of antibiotic resistant esculin-hydrolyzing bacteria from river-dependent and groundwater-dependent tilapia farms in Pampanga, Philippines. Esculin-hydrolyzing bacteria showing high rate of resistance belong to coliform group and considered as indicator of antibiotic pollution. Generally, isolates from river-dependent farms exhibited higher rate of resistance to some antibiotics tested across sources (water, sediment and fish) than their groundwater-dependent counterparts. Meanwhile, antibiotics such as chloramphenicol, ciprofloxacin, nitrofurantoin, norfloxacin and tetracycline can still effectively inhibit esculin-hydrolyzing bacteria, specifically the Gram-negative ones. However, MAR index indicated that selected isolates from both farm types are resistant to multiple types of antibiotics. From this result, river-dependent and groundwater-dependent farms in Pampanga are already contaminated with antibiotic compounds and can be considered high-risk areas. Moreover, the isolates were identified to be potentially pathogenic and clinically important. Based on the result of sequencing, the multi-antibiotics resistant esculin-hydrolyzing bacteria isolated from this study belong to the genera of *Aeromonas*, *Enterobacter*, *Klebsiella*, *Kosakonia*, *Phytobacter*, and *Superficieibacter*. Of these, *Klebsiella* sp. dominate in river-dependent farms while *Aeromonas* sp. are common in groundwater-dependent farms. The presence of multi-antibiotic bacterial strains could pose serious threats in tilapia production as well as the health of consumers. It is crucial to identify the primary source of antibiotic contamination among farm areas. The source of water could not be a major factor for the emergence of multi-antibiotic resistant bacteria as there is comparable risk between river-dependent and groundwater-dependent farms. Further studies are essential for better understanding of the dynamics of antibiotic resistance among these bacteria to develop a concrete surveillance system in the future.

Conflict of interest. The authors declare that there is no conflict of interest.

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