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Emergence of Antibiotic Resistance Genes sul1, tetA, bla_{GES}, and mexF in Sapon Irrigation Canal and Aquaculture Pond in Kulon Progo Regency, Indonesia

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ABSTRACT

Antibiotic resistance genes (ARGs) have recently become an emerging environmental contaminants. The aquatic environment, such as a river has already become the most polluted environment and can be a driver of ARGs. The water from irrigation canal has the potential to become a hotspot of ARGs through contamination from river pollutants carried along to the irrigation canal. However, the information regarding the cross-contamination of ARGs in fish farming systems integrated with irrigation canal in Indonesia needs further study. This study investigated the occurrence of ARGs sulfonamide (*sul1*), tetracycline (*tetA*), beta lactam (bla_{GES}), and multi drug resistance (*mexF*) from body water samples along the irrigation canal and aquaculture ponds which utilize irrigation water for cultivation. Sampling sites are located in the Kulon Progo Regency (Indonesia) and samples were taken during the rainy season. Gene amplification was performed using Multiplex PCR. The results showed that *sul1*, *tetA*, and bla_{GES} were detected in 67%, 63%, and 55% of all samples. Meanwhile, *mexF* was only found upstream and downstream irrigation canals, which accounted for 25% of the total samples. The results of this study indicated that the Sapon Irrigation Canal has the potential to cause the spread of antibiotic resistance genes.

Keywords: antibiotic resistance gene, aquaculture, aquatic environment, irrigation canal, multiplex PCR.

INTRODUCTION

Antibiotic resistance has become one of the greatest threats to global health and food security. This condition can reduce the effectiveness of bacterial infection treatment. Murray et al. (2022) reported that in 2019, the cases of deaths associated with antibiotic-resistant bacteria were predicted to reach 4.95 million, including 1.27 million deaths directly caused by antibiotic-resistant bacteria. The main cause of antibiotic resistance is the inappropriate use of antibiotics in human and veterinary medicine, as well as in food production. Most antibiotics are not metabolized, and when excreted from the body with urine and feces to the environment, these compounds are still in active form (Zhou et al., 2021).

Antibiotics have recently been classified as pseudo-resistant contaminants because they are continuously introduced into the environment, but are very difficult to degrade (Kulik et al., 2023). The presence of these contaminants in the environment needs to be monitored, especially their potential to cause the spread of antibiotic resistance (Koch et al., 2021). This group of contaminants includes antibiotic-resistant bacteria

(ARB), antibiotic-resistance genes (ARG), mobile genetic elements (MGE), and antibiotic residues (Larsson & Flach, 2022). Naturally, the antibiotic resistance traits found in bacteria are encoded by the genes located on chromosomes or plasmid components. The existence of antibiotic-resistance genes (ARGs) in the environment needs to be monitored because they can be transferred between bacteria, both non-pathogenic and pathogenic, through a horizontal gene transfer (HGT) mechanism, which can increase the risk of infection due to pathogenic bacteria that are resistant to several types of antibiotics, known as superbugs (Urban-Chmiel et al., 2022).

The aquatic environment can be a source of various types of infectious bacteria that have the opportunity to disseminate antibiotic resistance. Sanitation and water pollution are important factors in the spread of antibiotic resistance. Rivers are aquatic ecosystems that have been widely reported to be reservoirs for various kinds of contaminants such as nutrients, agrochemicals, metals, antibiotic residues, and personal care products of different origins (agriculture, cities, and industry) as well as play a major role in antibiotic resistance drivers (Muurinen et al., 2022). Nonetheless, rivers are widely used for various human activities, such as irrigation. Irrigation canals play an important role in supporting food production (agriculture and aquaculture). This condition may cause cross-contamination of ARGs carried by irrigation water to food produced. In agriculture, the use of irrigation water can cause the transfer of ARB and ARGs, especially to the food that can be consumed raw (Amato et al., 2021). Meanwhile, in aquaculture, the presence of ARB and ARGs can affect fish disease management and also has the potential to cause food-borne disease through HGT, which can spread among aquatic microbial communities and reach human pathogenic bacteria (Pepi & Focardi, 2021).

In Indonesia, many fish farmers are using irrigation canals as the source of water for their aquaculture activities. However, there are still few reports regarding the spread of antibiotic resistance in such aquaculture systems. Limited information regarding the spread of antibiotic resistance in fisheries activities in Indonesia also causes a lack of scientific evidence that can explain this issue. On the basis of this problem, this research aimed to investigate the distribution of antibiotic resistance genes along the irrigation canal, which is also used for aquaculture activities, by using Galur Sub-district, Kulon Progo Regency, Special Region of Yogyakarta as the location examples. This fundamental foundation is expected to be supporting data for the handling of antibiotic resistance in Indonesia, which is implemented in the National Action Plan for Antimicrobial Resistance Mitigation for 2020–2024, especially those related to the fisheries sector.

MATERIALS AND METHODS

Sampling location and sample collection

The sampling was done in the Irrigation Canal, Galur Sub-district, Kulon Progo Regency, Special Region of Yogyakarta. The source of water that enter the irrigation canal comes from the Progo River. Sampling was carried out at 5 location points along the Sapon Irrigation Canal which 2 locations were used for aquaculture activities (Table 1 and Figure 1). This location consists of the intake dam (D), fish pond A (FPA), fish pond B (FPB), outlet dam (O), and estuary (E)**.** The river water that enters the irrigation canal was collected from the intake dam (D). After this point, most of the irrigation water will be channeled into the irrigation network then used for agricultural and fisheries activities. Fish ponds were then divided into 3 sampling points consist of fish pond inlet (FPAI/FPBI), fish pond (FPAP/FPBP), and fish pond outlet (FPAO/FPBO). Most of the irrigation water will go to the dam outlet canal (O) before flowing into the estuary (E) and being discharged into the sea. Sampling was carried out in the period January 2023 – March 2023, which coincides with the rainy season. At each location point, as much as 500 ml of samples were taken aseptically with 3 replicates. The water samples were then placed into icebox for immediate processing at the laboratory.

DNA extraction

The samples of irrigation and fish pond water were filtered with a vacuum pump with polyethylene sulfone (PES) membrane filters 0.2 µm 47 mm. Genomic DNA was isolated by using DNeasy PowerWater Kit (Qiagen) with the manufacturer's recommendations.

Antibiotic resistance gene detection

The presence of antibiotic resistance genes in the collected samples was determined by PCR

Table 1. Sampling location

Sampling location	Latitude	Longitude
Intake dam (D)	-7.922642°	110.255087°
Fish pond A (FPA)	-7.946272°	110.221717°
Fish pond B (FPB)	-7.92806°	110.182601°
Outlet dam (O)	-7.97778°	110.205233°
Estuary (E)	-7.979689°	110.209287°

assay. Specific primers for four ARG, related to different families of antibiotics, were selected (Table 2): *sul1* (sulfonamide), *tetA* (tetracycline), bla_{GES} (beta lactam), and *mexF* (multidrug resistance). The selection of ARG is based on their presence in aquatic environment (Liang et al., 2020; Teixeira et al., 2020; Amato et al., 2021; Lye et al., 2022; Muurinen et al., 2022). Moreover, Muurinen et al. (2022) reported that these genes are the most abundant from their antibiotic group and distributed along the Code River, Indonesia. The PCR reaction mix had total volume of 25 µl which contain PCR Master Mix (MyTaq HS Red Mix) 12,5 µl, 10 µM each primer 1 µl, template DNA 1 µl, and ddH_2O. The PCR reaction was started with denaturation at 95°C for 2 min followed by 30 cycles for 1 min 30 s at 95°C, annealing temperature at 63°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min. PCR products were detected by electrophoresis on 1.8% (w/v) agarose gel in TBE 1X buffer with the addition of DNA stain (GelRed) at 110 V for about 30 min and visualized by UV.

RESULTS AND DISCUSSION

The Sapon Irrigation Canal has an area of ±2094 Ha which flows into three sub-districts in Kulon Progo Regency, one of which is Galur Sub-district. This area is located in the southeast of Kulon Progo Regency and several residents use the irrigation canal for fish cultivation. In this study, the ARGs consisting of *sul1*, *tetA*, bla_{GES}, and *mexF*. *sul1* (sulfonamide) and *tetA* (tetracycline) were targeted; they are the most commonly ARGs found in environment and have become the environment indicators of ARGs contamination (Bourdonnais et al., 2022; Venkatesan et al., 2023). bla_{GES} (beta lactam) often found in hospital wastewater (Conte et al., 2022). Meanwhile, *mexF* (multi drug resistance) is a transporter protein in the multidrug efflux pump found in *Pseudomonas aeruginosa* which came from clinical isolates (Ozer et al., 2012). *P. aeruginosa* is opportunistic pathogenic bacteria which also can infect fish (Ali et al., 2021).

Rivers are lotic ecosystems that act as reservoirs for various types of pollutants, especially antibiotic which can also become vehicles for

Figure 1. Location of sampling sites

Table 2. PCR primer sequences

Target gene	Sequence	Product Size (bp)
sul1	5'-AGGCTGGTGGTTATGCACTC-3'	419
	5'-GAGAAGGTGATTGCGCTTCG-3'	
tetA	5'-TGACGGGCTGTTTCCTTTTG-3'	458
	5'-CTGCCTGGACAACATTGCTT-3'	
bla_{GES}	5'-CGGTTTCTAGCATCGGGACA-3'	321
	5'-CGTTTGGTTTCCGATCAGCC-3'	
mexF	5'-CCCAATTCTTCATCCAGCGG-3'	736
	5'-AACTCTTCCTCGGTGACCAG-3'	

these compounds to reach various locations. Antibiotic contamination is often found in the environment while still in the active phase at lethal doses which can trigger the development of resistant traits in aquatic microbial communities. This mechanism is also influenced by the presence of antibiotic-resistance genes (ARGs) which can spread between bacteria through horizontal gene transfer (HGT) mechanism. The results of ARGs detection in this study are shown in Table

Table 3. Antibiotic resistance gene detection

Samples		Antibiotic Resistance Genes			
		sul1	*tetA*	bla_{GES}	*mexF*
Intake dam (D)	D1	+	+	+	+
	D2	+	+	+	+
	D3	+	+	+	+
Fish pond A – inlet (FPAI)	FPAI1	n.d	n.d	n.d	n.d
	FPAI2	n.d	n.d	n.d	n.d
	FPAI3	n.d	n.d	n.d	n.d
Fish pond A – pond (FPAP)	FPAP1	+	+	+	-
	FPAP2	+	+	+	-
	FPAP3	+	+	+	-
Fish pond A – outlet (FPAO)	FPAO1	+	+	-	-
	FPAO2	+	+	+	-
	FPAO3	+	-	-	+
Fish pond B – inlet (FPBI)	FPBI1	n.d	n.d	n.d	n.d
	FPBI2	n.d	n.d	n.d	n.d
	FPBI3	n.d	n.d	n.d	n.d
Fish pond B – pond (FPBP)	FPBP1	+	+	+	-
	FPBP2	+	+	+	-
	FPBP3	+	+	-	-
Fish pond B – outlet (FPBO)	FPBO1	+	+	+	-
	FPBO2	+	+	+	-
	FPBO3	+	+	+	+
Outlet dam (O)	O1	+	+	+	+
	O2	+	+	+	+
	O3	+	+	+	-
Estuary (E)	E1	n.d	n.d	n.d	n.d
	E2	n.d	n.d	n.d	n.d
	E3	n.d	n.d	n.d	n.d

Note: *n.d = not detected.

3. From the river water samples which were taken at the intake dam (D), it can be seen that all of the tracked ARGs (*sul1*, *tetA*, bla_{GES}, *mexF*) in this study were found. Various reports have proven the existence of *tetA* (Liang et al., 2020; Muurinen et al., 2022; Zhang et al., 2022), *sul1* (Lye et al., 2022; Muurinen et al., 2022), bla_{GES} (Teixeira et al., 2020; Muurinen et al., 2022), and *mexF* (Muurinen et al., 2022) in river waters. Antibiotic contamination in waters can come from improper handling of pharmaceutical industry and hospital drug waste, the use of antibiotics in livestock and fisheries activities, the application of manure for agricultural activities, as well as the presence of antibiotic residues carried in the urine and feces of humans and domestic animals (Larsson & Flach, 2022). These findings indicate that the water entering the Sapon Irrigation Canal has the potential to cause the spread of antibiotic resistance in the food production chain in Kapanewon Galur, Kulon Progo Regency.

After the river water enters the intake dam, it will distribute into the irrigation network for agriculture and aquaculture purposes. In this study, the irrigation water used for aquaculture was taken from 2 locations. The utilization of irrigation water for aquaculture was divided into 3 groups based on cultivation step. This group consists of fish pond intake (FPAI and FPBI), fish pond (FPA and FPB), and fish pond outlet (FPAO and FPBO). On the basis of the results of antibiotic resistance gene detection in Table 3, it can be seen that in the fish pond inlet samples all of ARGs were not detected (n.d). The non-detection of this gene is likely due to movement in the irrigation canal water flow. This condition is can be related to DNA concentration amount from this samples which is only in the range of 0.62–0.69 ng/µl (FPAI) and 0.61–0.71 ng/µl (FBAI). Low DNA concentration may cause by flowing water which can affect the distribution of environmental microorganism including the presence of ARGs. This condition causes a lack of nutrient availability for microorganisms to carry biological activities. Biofilm formation also plays role in overcoming environmental stress conditions and protecting cells due to water current (Lin et al., 2021). However, the information regarding the distribution of ARGs in aquatic environment was still limited. The available evidence only shows that the movement of ARGs are strongly influenced by the presence of solid particles and biofilms (Lin et al., 2021; Joseph et al., 2022).

The *tetA*, *sul1*, and bla_{GES} are detected in both fish pond locations (FPA and FPB). On the basis of the fish farmer's explanation, no antibiotics are used during cultivation to cure disease. When a disease infection occurs, they tend to harvest early to avoid losses. This condition was similar in all cultivation pond locations. Fish ponds are closed systems that allow for accumulation of organic material from feed residues and feces which can used as nutrients for microorganisms. The availability of these nutrients allows bacteria to undergo replication to increase the presence of ARGs in fish pond samples. Fish feed has potency to become ARGs reservoir (Han et al., 2017), but even it does not contain antibiotics, it can still promote accumulation of ARGs by provided nutrients for microrganisms (Han et al., 2018; Raza et al. 2022). Fish feed can promote the spread of tetracycline (*tetD*; *tetX*), sulfonamides (*sul1*; *sul2*), and beta lactam (bla_{TEM}) resistance genes during fish cultivation. The use of probiotic also can cause antibiotic resistance spreading in aquaculture pond (Anokyewa et al., 2021). Both fish feed and probiotics are very important to support aquaculture. However, this combination has great potency to cause the spread of antibiotic through fish cultivation. It because each bacteria (non-pathogen and pathogen) can carry HGT to develop resistant traits and will become a threat if this happens to human pathogen bacteria.

Besides that, tetracycline (oxytetracycline, chlortetracycline, and tetracycline) and sulfonamide (sulfadiazine) are a group of antibiotic which still permitted to use in the fisheries sector according to Ministerial Regulation of Maritime Affairs and Fisheries of the Republic of Indonesia Number 1 of 2019 concerning Fish Medicine. The presence of the *sul1* and *tetA* genes in fish pond is an indication that there is a possibility the use of tetracycline and sulfonamide antibiotics will be ineffective for curing fish disease and may increase antibiotic resistance in the microbial community in the pond and fish's digestive tract itself. bla_{GES} also detected on most of the samples. This gene is often found in hospital wastewater (Conte et al., 2022), and various types of water environment, such as rivers (Teixeira et al., 2022; Muurinen et al., 2022) and waste water treatment plants (WWTPs) (Fadare & Okoh, 2021; Waśko et al., 2022). The presence of this gene from clinical bacteria contamination also needs special

attention since it can have impact on bacterial infection which can affect the fish farmer.

The presence of ARGs on fish pond outlet samples (FPAO and FPBO) was not much different than fish ponds samples. It is because these samples originate from a waste water pond which is routinely released to reduce the accumulation of organic compounds which can affect the quality of fish cultivation. This waste water will enter the drainage canal which flowing to the outlet canal before enter to the estuary and sea.

Accumulation of waste water carried by irrigation canal will flow to the dam outlet which is located in vicinity of the Progo River estuary. All of ARGs are detected in water samples that taken from dam outlet (O). *sul1*, *tetA* and bla_{GES} are the most common ARGs detected from all of the samples along the irrigation canal. The results showed that these genes were detected in 67%, 63%, and 55% of all samples respectively. *sul1* and *tetA* recently been determined to be indicators of antibiotic contamination in the environment (Bourdonnais et al., 2022; Venkatesan et al., 2023). According to Siri et al. (2023), *sul1*, *tetA*, and bla_{TEM} are the most commonly detected in ARGs from wastewater, freshwater, and salt water sample in Southeast Asia. In this study the presence of *mexF* only detected on dam intake (D) and dam outlet. Lai et al. (2021) reported that from 23 ARGs related to multi drug resistance (MDR), *mexF* is the most abundance gene detected in downstream sites at Eskilstuna, Stockholm, and Västerås, Sweden. Muurinen et al. (2022) also reported that *mexF* is the most abundance gene in downstream sites and estuary along the Code River, Yogyakarta, Indonesia. The findings of these ARGs contamination indicate that irrigation canal has the potential to cause the spread of antibiotic resistance and have great risk of impacting both agriculture and fisheries farmers.

After flowing along the irrigation canal, most of irrigation water will be discharged into the estuary and sea. In this study, all ARGs were not detected (n.d) in the estuary (E), even *sul1*, *tetA,* and bla_{GES} which are the most presence ARGs. This condition may cause by the mixing event between fresh water and sea water in the estuary which cause a dilution process which results in decrease of the DNA concentration obtained (Supplement 1) and affect PCR amplification. This low concentration of DNA has the potential to cause false negatives in the analyzed samples (Curtis et al., 2021; Kestel et al., 2022).

CONCLUSIONS

Irrigation canals have the potential to be a means of spreading ARGs. The *sul1*, *tetA*, and bla_{GES} genes were the most prevalent ARGs, which were detected in most of the samples. The results showed that these genes were detected in 67%, 63%, and 55% of all samples, respectively. However, *mexF* was only found in locations of dam intake and dam outlet, by 25% of samples. The existence of the *tetA* and *sul1* genes requires special attention, since these two genes play a role in the emergence of resistance to tetracycline and sulfadiazine antibiotics, which can still be used as fish medicine in the fisheries sector.

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