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Antibiotic resistance of emerging pathogenic bacteria of hybrid grouper farming in Indonesia

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Abstract. *Istiqomah I, Isnansetyo A, Murwantoko, Handayani DP, Lestari YN, Taslihan A, Permana IGN, Wijayanti E. 2023. Antibiotic resistance of emerging pathogenic bacteria of hybrid grouper farming in Indonesia. Biodiversitas 24: 2493-2501.* A hybrid grouper is among the main coastal farming species in Asian countries, yet vibriosis is still a significant issue. Therefore, a knowledge of pathogenic strains responsible for recent cases is essential for developing appropriate control measures. The purpose of the study was to describe emerging bacterial pathogens associated with hemorrhagic disorder, skin wounds, and mortality in *cantang* hybrid grouper farms in Indonesia. A total of 30 bacterial strains were recovered from the kidney of diseased grouper on TCBS agar. Based on the ability to induce clinical signs and mortality of grouper juveniles owing to intraperitoneal bacterial infection at 10^6 CFU/fish, the bacteria were divided into three groups: pathogenic (n=11), low-pathogenic (n=2), and non-pathogenic (n=17). The pathogenic strains were putatively identified as *Photobacterium damselae* subsp. *Damselae* (n=8), *Vibrio alginolyticus* (n=1), *Vibrio harveyi* (n=2), and *Vibrio azureus* (n=2) based on the biochemical characteristics and 16S rRNA and DNA gyrase B subunit gene sequences. The pathogenic bacteria were sensitive to erythromycin, gentamycin, oxytetracycline, and kanamycin. However, it still has various resistant patterns to ampicillin, fosfomycin, chloramphenicol, streptomycin, rifampicin, and enrofloxacin. This study demonstrated the emergence of pathogenic *P. damselae* subsp. *damselae*, *Vibrio alginolyticus*, *V. harveyi*, and *V. azureus* in grouper aquaculture in Indonesia with multiple antibiotic resistance, which needed to further studies.

Keywords: 16S rDNA, antibiotic-resistant, DNA gyrase, vibriosis, *Vibrio alginolyticus*, *Vibrio harveyi*

INTRODUCTION

Grouper is a significant mariculture species in Asia and the Mediterranean (Cabaleiro et al. 2018; Yan et al. 2020). As a result, hybrid grouper has become the most popular species in Asia (Arrokhman et al. 2017; Yan et al. 2020). However, there have been multiple reports of regular disease outbreaks in hybrid *cantang* grouper (♀ *Epinephelus fuscoguttatus*×♂ *Epinephelus lanceolatus*) farms in Indonesia, with similar clinical signs of skin hemorrhage and necrosis (Koesharyani and Zafran 2017; Mahardika et al. 2020). In addition, the same cases have been recorded in Malaysia (Mohamad et al. 2019) and China (Zhu et al. 2018).

Vibriosis is one of the emerging diseases in Asian grouper aquaculture (Zhang et al. 2022). More than 85 *Vibrio* species are responsible for vibriosis (Baker-Austin et al. 2018), 14 of which have been identified in Indonesia (Istiqomah et al. 2020). The causative agents of vibriosis shift irregularly, challenging control (Ina-Salwany et al. 2019). In shrimp cultivation, for example, emerging *Vibrio parahaemolyticus* (Yingkajorn et al. 2014) and *Vibrio campbellii* (Dong et al. 2017; Nuidate et al. 2021) have surpassed luminescent *Vibrio harveyi* (Chrisolite et al. 2008). However, *Vibrio harveyi* remains prevalent in marine fish farming (Mougin et al. 2021). *Photobacterium damselae* subsp. *damselae (Vibrio damsela)* is a multiclonal pathogen with considerable genetic diversity that has recently emerged in marine fish aquaculture, most notably in rainbow trout, *Oncorhynchus mykiss* (Walbaum) (Gouife et al. 2022). This pathogen dominated the spatial pattern found in snapper (*Lates calcarifer*) culture, along with *Vibrio alginolyticus*, *V. harveyi*, *Vibrio owensii*, and *Vibrio rotiferianus* (Mougin et al. 2021). It is believed that the ability of various virulence factors to adapt (Castillo et al. 2018; Mohamad et al. 2019), as well as massive outbreaks of antibiotic resistance lateral gene transfer (Mohammad et al. 2019; Hoihuan et al. 2021; Yan et al. 2020), is linked to the spread of these emerging pathogens.

Antibiotic resistance in aquaculture is important to mitigate because it can potentially spread resistant microbes to seafood products and the environment worldwide. Antibiotics are being used in modern aquaculture due to their need for high yields (Miller and

Harbottle 2018). However, fish farmers frequently misuse antibiotics to control disease without first determining the type of disease-causing agent (Shao et al. 2020) or to avoid disease by regularly adding it to fish feed (Zhou et al. 2018). Due to these practices, antibiotic residues are present in aquaculture products and the environment, serving as a source of lateral antibiotic resistance gene transfer among aquatic microbes (Chuah et al. 2016). Although little is known about the antibiotic susceptibility of emerging pathogenic *Vibrio* spp. of mariculture in Indonesia, multiple antibiotic-resistant *V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus*, and *P. damselae* subsp. *damselae* strains appear to be on the rise globally (Miller and Harbottle 2018; Matamp and Bhat 2019; Isnansetyo et al. 2022; Petchimuthu et al. 2022). The antibiotic resistance of these emerging pathogens has become a particular stress for the aquaculture industry, particularly in tightening antibiotic use and suggesting other health management methods such as vaccines, immunostimulants, and probiotics. Since the 1990s, vibriosis has been an issue in Indonesian aquaculture. Random cases continue to emerge despite the use of commercial vaccines and different control techniques (Istiqomah et al. 2020). This raises the question of whether or not a novel pathogen caused those instances. Accordingly, this research was initiated to accurately depict the type and characteristics of the current pathogens causing the disease outbreak of hybrid grouper farming in Indonesia and investigate their susceptibility to available antibiotics.

MATERIALS AND METHODS

Fish sampling and bacterial isolation

Fish sampling was undertaken from the hybrid grouper mariculture areas that were reported to have disease outbreaks in Sekupang (Batam), Bulu (Central Java), Panarukan (East Java), and Gerokgak (Bali), Indonesia, in 2017-2022 (Figure 1). Fish with external disease signs such as hemorrhagic, skin lesion, necrosis, fin rod, and moribund stage were selected for bacterial isolation. A total of 25 juveniles and 15 subadult grouper from the hatchery and floating cage net were collected and dissected for bacterial isolation from the kidney. While sampling, the water's dissolved oxygen concentration, and pH were examined using a water quality checker (Horiba). At the same time, the total vibrio content (colony forming unit count) was confirmed on Thiosulfate Citrate Bile Salts Sucrose(TCBS) agar (Oxoid). Bacteria were inoculated onto TCBS agar and incubated at room temperature for 48 hours for bacterial purification on the same agar. Colonies from the TCBS plate were purified on Tryptone soya agar (Oxoid) medium supplemented with NaCl (3% w/v; TSAS) at room temperature followed by stored in tryptone soya broth (Oxoid) medium supplemented with NaCl and glycerol (3% w/v and 25% v/v; TSBSG) in-80^{o}C for further identification.

Artificial infection of isolated bacteria

A fry of grouper measuring 4-5 cm in length was selected for the bacterial infection experiment. The Postulate Koch test was performed in 10 fish/bacteria by intraperitoneal injection of purified bacteria at 10^6 CFU/fish. The control fish received a 0.01 mL injection of physiological saline. The infected fish were kept in ozonized sea water with aeration but no feeding. Fish mortality was observed up to ten days after infection. The dead fish was dissected to isolate bacteria from the kidney.

Biochemical and physiological characterization of the pathogenic bacteria

A freshly prepared bacterial culture (24 h) was used for biochemical and physiological characterization. In addition, a standard Gram staining, oxidase, catalase, OF test, and sensitivity to 2,4-diamino-6,7-diisopropyl pteridine phosphate (O/129) test based on the MacFaddin (2000) were conducted. The Rapid Biochemical Test based on HiVibrio™ Identification Kit (KB007, Himedia) was used for further bacterial characterization.

16S rRNA and DNA gyrase B subunit gene analysis of the pathogenic bacteria

The bacterial isolates' genomic DNA was prepared using Wizard genomic DNA isolation kit (Promega). The isolated genome was then amplified using two sets of universal primers for the 16S rRNA gene, 27F (5-AGAGTTTGATCMTGGCTCAG-3) and 1492R (5-TACGGTTACCTTGTTACGACTT-3) (Isnansetyo and Kamei 2003) or DNA gyrase B subunit gene, UP-1 (5-GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNA ARTTY-3) and UP-2r (5-AGCAGGGTACGGATGTGCG AGCCRTCNACRTCNGCRTCNGTC-3) (Yamamoto and Harayama 1995). The PCR products were developed in a 1.5% gel agarose electrophoresis, followed by agarose gel DNA extraction. Gene sequencing was done with ABI®PRISM® Big dye terminator v3.1 cycle sequencing kit (Applied Biosystems) and ABI 3130 Genetic Analyzer (Applied Biosystems).

Homology search of nucleotide sequence obtained from the sequencing process was done using Basic Local Alignment Search Tool (BLAST) algorithm program (Altschul et al. 1990). Then, the sample gene sequences were aligned with reference sequences from the BLAST results. Next, samples with a more than 97% similarity value were observed using Clustal W Multiple Alignment in the Molecular Evolutionary Genetics Analysis (MEGA) application version 10.0 to create a neighbor-joining phylogenetic tree with bootstraps of 1000 replicates.

Antibiotic susceptibility test

Pathogenic bacteria in the present study were tested on their susceptibilities to antibiotics based on the previously described disc diffusion method (CLSI 2006). Each bacterial strain was cultured on TSA overnight and diluted with PBS for adjustment of turbidity to 0.5 McFarland and spread on Muller-Hinton agar plates (100 um/plate). Antibiotic disc (Advantec) containing: ampicillin (AMP, 25 μg), chloramphenicol (CHL, 30 μg), enrofloxacin (ENR, 25 μg), erythromycin (ERY, 10 μg), gentamicin (GEN, 10 μg), fosfomycin (FOS, 200 μg), kanamycin (KAN, 30 μg), oxytetracycline (OXY, 10 μg), Rifampicin

(RIF, 5 μg) and streptomycin (STR, 254 μg) were arranged on top of the agar plates for overnight incubation in room temperature. The inhibition zone diameter was measured to determine the sensitivity against the antibiotic based on CLSI guidelines (CLSI 2014). The antibiotic resistance pattern of the pathogenic isolates was used to calculate the multiple antibiotic resistance index (MARI) according to the previous study (Gxalo et al. 2021).

RESULTS AND DISCUSSION

Bacterial isolation and infection test

A total of 30 bacterial isolates with a greenish-yellow to yellow appearance on TCBS agar were recovered from diseased fish with symptoms of skin necrosis, fin necrosis, skin hemorrhagic, wound on the skin, and lethargies (Figure 1, Table 1). Yellow colonies grew on TBCS appear as the dominant bacterial type over another greenish one.

The water quality of grouper aquaculture in all fish farms in this study was in good condition (Table 1). Water in fish hatcheries has a dissolved oxygen content of 5.1-6.3 mg/L, a pH of 7.0-7.7, and log total presumptive *Vibrio* of 2.2-3.6 cfu/mL. The pond water dissolved oxygen content, pH, and log total presumptive *Vibrio* were 6.0 mg/L, 7.7, and 2.7 cfu/mL. Meanwhile, sea cage culture water observed in this study has a dissolved oxygen content of 5.8-6.9 mg/L, pH 7.7-7.8, and log total presumptive *Vibrio* of 2.2-2.7 cfu/mL.

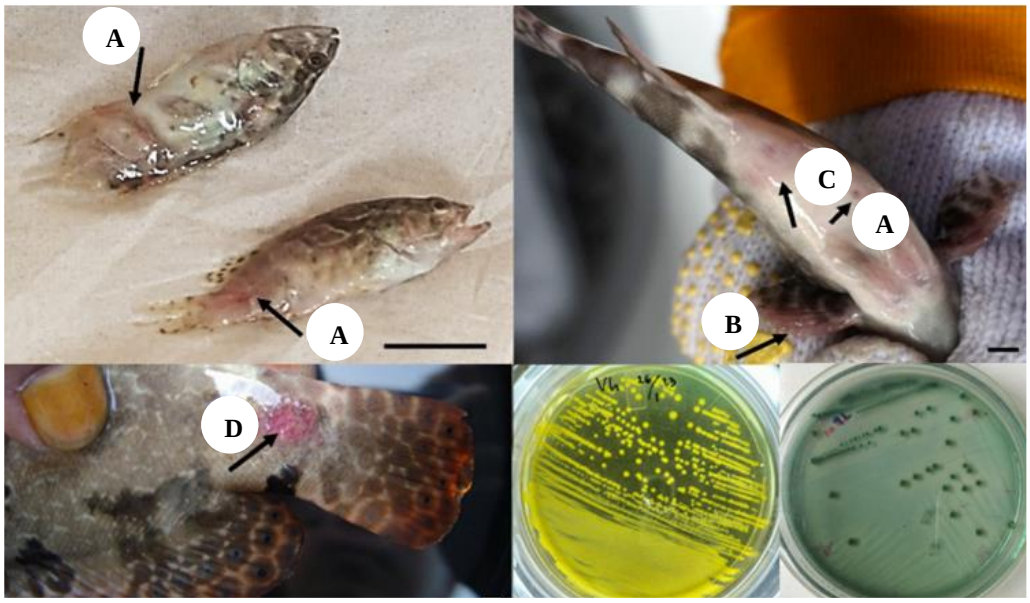

Figure 1. Sample of diseased *cantang* hybrid grouper with skin necrosis (A), fin necrosis (B), skin hemorrhagic (C), and wound on the skin (D) with the bacterial colonies obtained from the fish kidney. Bar = 1 cm

Table 1. Water quality and the number of pathogenic bacterial strains isolated from mariculture farms in Indonesia

Sampling site	Water dissolved oxygen (mg/L)/pH/salinity (ppt)/temp. °C	Vibrio count in water (log cfu/mL)	Num. of isolates (pathogen/total)	Pathogenic isolates (percent of challenge fish mortality, mean time to death in days post-infection)*
Batam				
Hatchery	5.1/7.7/33/29	2.5	0/1	-
Cage net 1	6.1/7.7/33/29	2.2	0/1	-
Cage net 2	5.8/7.8/33/29	2.3	0/1	-
Central Java				
Fish pond	6.0/7.7/33/29	2.7	2/7	***Vaz*** JP01 (100,1), ***Vaz*** JP07 (100,1)
East Java				
Hatchery 1	5.3/7.3/33/29	3.5	2/4	***Pdd*** SB01 (100, 1), ***Pdd*** SB06 (100,1)
Hatchery 2	5.4/7.3/33/29	3.0	1/3	***Vh*** SB25 (60, 1)
Cage net	6.2/7.8/33/29	2.7	2/3	***Pdd*** SB26 (100, 1). ***Pdd*** SB22 (100,1)
Bali				
Hatchery1	5.0/7.4/33/29	2.9	2/3	***Pdd*** GD05 (100, 2), ***Pdd*** GD06 (100, 2)
Hatchery2	6.3/7.0/33/29	3.6	2/4	***Pdd*** GD09 (100, 1), ***Val*** GD22 (100, 6)
Cage net	5.9/7.7/33/29	2.4	2/3	***Pdd*** GD34 (100, 3), ***Vh*** GD38 (100, 1)
Total			13/30	

Note: *Vaz: *Vibrio azureus*, Pdd: *Photobacterium damselae* subspecies *damselae*, Vh: *Vibrio harveyi*, Val: *Vibrio alginolyticus* according to Table 2 and Table 3

An artificial intraperitoneal infection indicated that 13 strains were pathogenic to grouper juveniles based on the ability to develop disease signs and kill the fish species at 100 or 60% of the population in five days post-infection (Table 1). During the infection test, disease symptoms such as hemorrhagic and necrotic of the fins base and skin, kidney swelling, and abdominal bleeding emerged. The symptoms produced by each of these pathogen isolates were mostly identical. Bacteria isolated from the kidneys of moribund fish produced colonies with the same morphology as the initially infected bacteria. Most of these pathogenic bacteria come from hatcheries (54%) and sea cages (31%), while only a small part comes from ponds (16%).

The isolation rate of pathogenic bacteria differs between locations (Figure 2). Pathogenic bacteria were commonly obtained from East Java and Bali, with recovery rates of 40% for *P. damselae* subsp. *damselae* and 10% for *V. alginolyticus* and *V. harveyi*. Meanwhile, pathogenic *V. azureus* were collected from diseased fish from the Central Java region with an isolation rate of 29%. Although samples of diseased fish from the Batam region had the same symptoms as those from other regions in the present study, no pathogenic bacteria were obtained.

Characterization and identification of pathogenic bacteria

Phenotypic characterization showed that all pathogenic bacteria in this study grew in TCBS agar medium and formed yellow or greenish-yellow colonies (Table 2). All of these pathogens have the distinctive characteristics of the genus Vibrio, such as being short rod-shaped, Gram-negative, motile fermentative in the OF test, containing catalase and oxidase enzymes, fermenting glucose without producing gas, and sensitive to Vibrio static agent (O/129). Biochemical test based on HiVibrio™ Identification Kit (KB007, Himedia) found that all strains were VP negative, able to grow on media with a NaCl content of 1-3%, and unable to use salicin as an energy source. In addition, most of these strains lacked beta-galactosidase (O-nitrophenyl-beta-D-galactopyranoside/ONPG negative), produced ornithine decarboxylase and were unable to use cellobiose as a carbon source. Meanwhile, various responses were shown by the bacterial strains in the arabinose, citrate, and sucrose utilization tests and arginine dihydrolase tests.

DNA isolation, PCR, and sequencing of the bacterial 16S rRNA and DNA gyrase B subunit genes resulted in DNA sequences sizing 874-1,345 bp and 1,045-1,365 bp, respectively (Table 3). A homology search of the genes with the BLAST program on the NCBI website found the proximity of the eight pathogenic bacteria (SB26, SB06, SB01, GD09, SB22, GD05, GD34, GD06) to *P. damselae* subsp. *damselae*, with 86%-100% query coverages and 99.2%-100% identities. Meanwhile, two strains (GD38 and SB25) had the highest homology to *V. harveyi*, with 100% query coverages and 99.4%-99.85% identities. Isolate D22 had the highest homology against *V. alginolyticus*, while isolates JP01 and JP07 had the highest similarity to *V. azureus*. The obtained sequences were multiple-aligned with reference genes for the cutting procedure in the same position and size, i.e., 870 bp and 1,052 bp for the 16S rRNA and DNA gyrase B subunit genes, to develop the phylogenetic tree (Figure 3).

Antibiotic resistance pattern of the pathogenic bacteria

The antimicrobial susceptibility of pathogenic bacteria in this study varied. All pathogenic strains were sensitive to erythromycin, gentamycin, oxytetracycline, and kanamycin, while some pathogens were resistant to other antibiotics (Figure 4). For example, in this study, *V. alginolyticus* strains were resistant to chloramphenicol, streptomycin, rifampicin, and enrofloxacin. In addition, two *V. harveyi* strains were resistant to ampicillin and enrofloxacin. Furthermore, *Photobacterium damselae subspecies damselae* were resistant to ampicillin (75% of the strains), chloramphenicol, and rifampicin (25%). Meanwhile, *V. azureus* resisted ampicillin (50% of the strains) and fosfomycin (100%).

Spatial variations in antibiotic resistance

Antibiotic resistance of pathogenic bacteria varied between locations (Figure 5). A total of two types of resistance were found from Central Java and East Java, while pathogens from Bali demonstrated five types of resistance. Pathogens from Central Java showed resistance to ampicillin (1 strain) and fosfomycin (2 strains). While, pathogens from East Java were resistant to ampicillin (4 strains) and chloramphenicol (1 strain). Pathogens from Bali showed resistance: to ampicillin (4 strains), chloramphenicol (2 strains), streptomycin (1 strain), rifampicin (3 strains), and enrofloxacin (1 strain). However, the multi-antibiotics resistance index (MARI) of pathogens from Central Java, East Java, and Bali were lower than 0.2 and not significantly different between locations ($p>0.05$). In addition, the MARI was higher in Bali (0.19) than in Central Java (0.13) and East Java (0.09) (Figure 6).

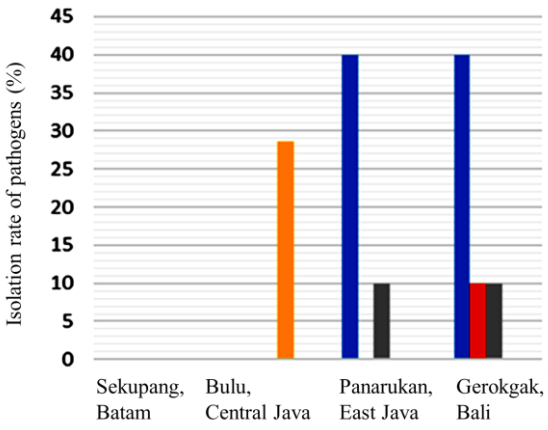

Figure 2. The isolation rate of pathogenic bacteria in each mariculture area in Indonesia. ■: *Photobacterium damselae* subsp. *damselae*; ■: *Vibrio alginolyticus;* ■: *V. azureus;* ■: *V. harveyi*

Table 2. Phenotypic characters of pathogenic *P. damselae* subsp. *damselae*, *Vibrio alginolyticus*, *Vibrio harveyi*, and *Vibrio azureus*

Characters	***Photobacterium damselae* subsp. *damselae****	***Vibrio alginolyticus*** **	***V. harveyi*** ***	***V. azureus*** ****
Colony in TCBS agar	Greenish yellow	Yellow	Yellow	Green
Luminescence	-	-	+	+
Cells shape	Short rod	Short rod	Short rod	Short rod
Gram	-	-	-	-
motility	+	+	+	+
Oxidation-fermentation test	Fermentative (F)	F	F	F
Oksidase	+	+	+	+
Catalase	+	+	+	+
Glucose utilization	+	+	+	+
Gas from glucose fermentation	-	-	-	-
Sucrose utilization	-(+)	+	+	-
Arabinose utilization	-(+)	-	-	+
Salicin utilization	-	-	-	-
Cellobiose utilization	-(+)	-	-	-
Arginine dihydrolase	+(-)	-	+	+
1-3% NaCl	+	+	+	+
Ornithine decarboxylase	+	-	+	-
Citrate utilization	-(+)	-	-	-
O-nitrophenyl-beta-D-galactopyranoside (ONPG)	-(+)	-	-	-
Voges Proskauer	-	-	-	-
Sensitivity to O/129	+	+	+	+

Note: *: SB26, SB06, SB01, GD09, SB22, GD05, GD34, GD06; **: GD22; ***: GD38, SB25; ****: JP01, JP07; +: all strains are positive; +(-): 80% of strains or more are positive; -: All strains are negative; -(+): 80% of strains or more are negative

Table 3. The length of the 16S rRNA and DNA gyrase B subunit genes sequences of the pathogens and the homology to reference

Pathogenic bacterial strains	16S rDNA			DNA gyrase B subunit		
	Length (bp)	BLAST (query coverage, identity)	Acc no. of reference	Length (bp)	BLAST (query coverage, identity)	Acc no. of reference
GD05	874	*Photobacterium damselae* subsp. *damselae* KC-Na27-SR1 (100%, 100%)	MN263233.1	1147	*Photobacterium damselae* V167.1 (100%, 99.8%)	LC37108.1
GD06	876	*Photobacterium damselae* strain TV27 (100%, 100%)	MT549173.1	1362	*Photobacterium damselae* subsp. *damselae* ATCC33559 (86%, 99.2%)	AY455889.1
GD09	874	*Photobacterium damselae* strain TV27 (100%, 99.54%)	MT549173.1	1365	*Photobacterium damselae* subsp. *damselae* ATCC33559 (86%, 99.4%)	AY455889.1
SB01	876	*Photobacterium damselae* strain QX175062 (100%, 99.89%)	MN310924.1	1358	*Photobacterium damselae* subsp. *damselae* ATCC33559 (87%, 99.4%)	AY455889.1
SB06	876	*Photobacterium damselae* strain TV27 (100%, 99.66%)	MT549173.1	1045	*Photobacterium damselae* subsp. *damselae* V1607-1 (100%, 99.81%)	LC370108.1
SB22	875	*Photobacterium damselae* strain TV27 (100%, 99.66%)	MT549173.1	1352	*Photobacterium damselae* subsp. *damselae* V1607-1 (100%, 99.78%)	LC370108.1
SB26	877	*Photobacterium damselae* strain AS-16-0963-3 (100%, 99.77%)	CP065041.1	1213	*Photobacterium damselae* ATCC33559 (100%, 99.84%)	AY455889.1
GD34	876	*Photobacterium damselae* strain TV27 (100%, 99.77%)	MT549173.1	1212	*Photobacterium damselae* ATCC33559 (100%, 99.67%)	AY455889.1
GD22	1179	*Vibrio alginolyticus* partial 16S rRNA gene (100%, 99.75%)	LN610441.1	1173	*Vibrio alginolyticus* DX0406 (100%, 99.91%)	EF579676.1
SB25	1345	*Vibrio harveyi* XSH1 (100%,99.78%)	MT071600.1	1356	*Vibrio harveyi* HQ050226-1 (100%, 99.6%)	GQ232761.1
GD38	1348	*Vibrio harveyi* XSHI (100%,99.85%)	MT071600.1	1355	*Vibrio harveyi* a661 (100%, 99.4%)	LR736262.1
JP01	1298	*Vibrio azureus* strain LC2-005 (100%, 99.92%)	NR_041683.1	1360	*Vibrio azureus* LC2-005 (100%, 99.78%)	CP018616.1
JP07	1296	*Vibrio azureus* strain LC2-102 (100%, 99.77%)	AB428898.1	1361	*Vibrio azureus* LC2-005 (100%, 99.85%)	CP018616.1

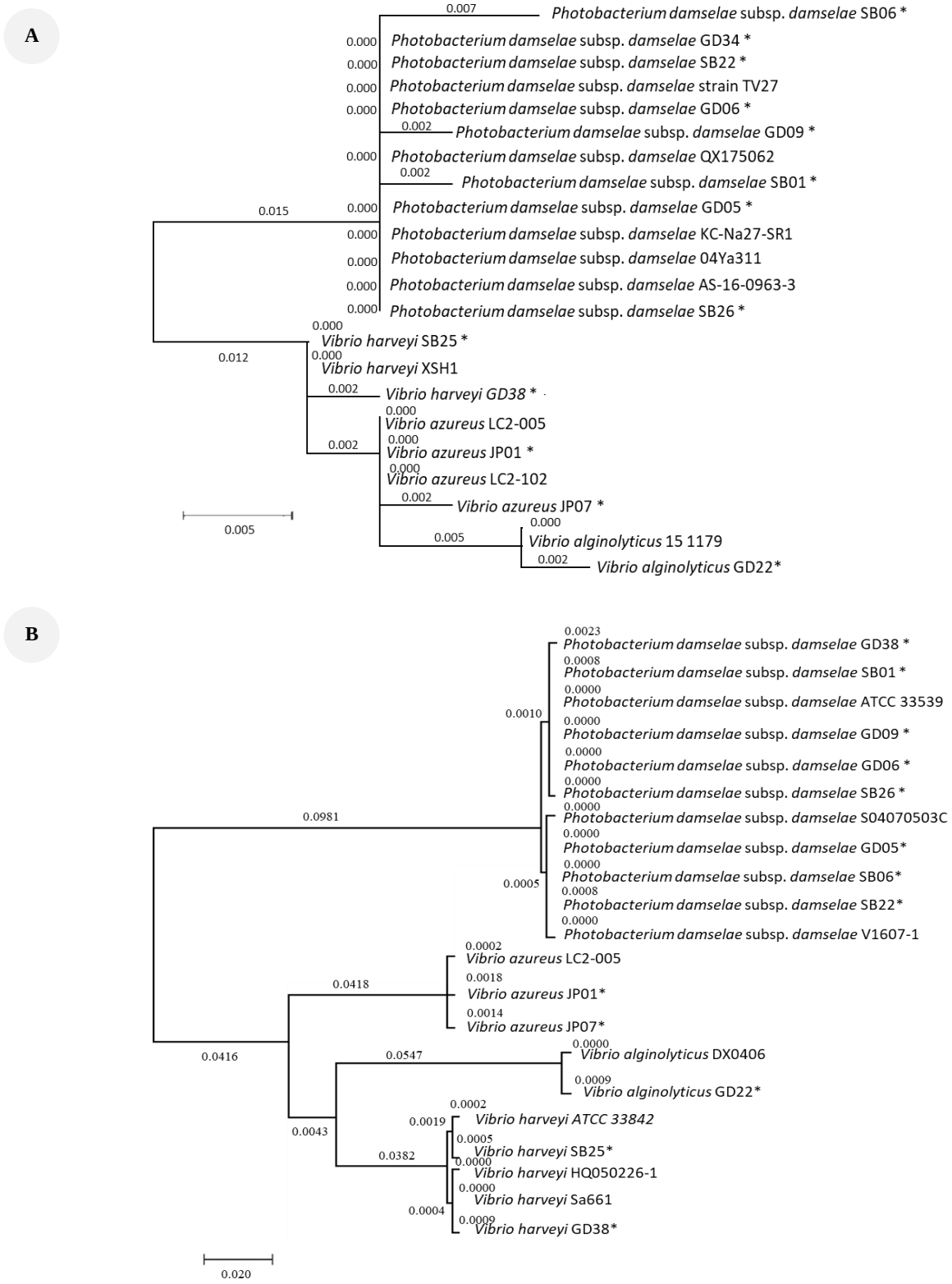

Figure 3. A. Phylogenetic tree of the 16S rRNA and; B. DNA gyrase B subunit genes of pathogenic bacteria in this study and reference bacteria in the Genbank using the Neighbour-joining method with 1,000 times bootstrapping. *: the strain of the present study

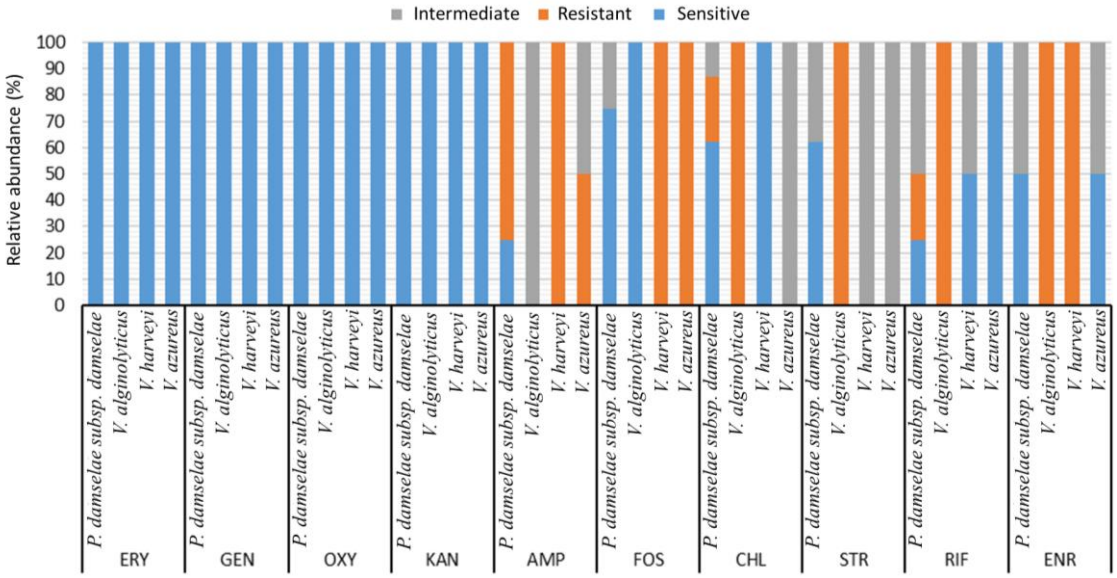

Figure 4. Antimicrobial resistance pattern of pathogenic strains

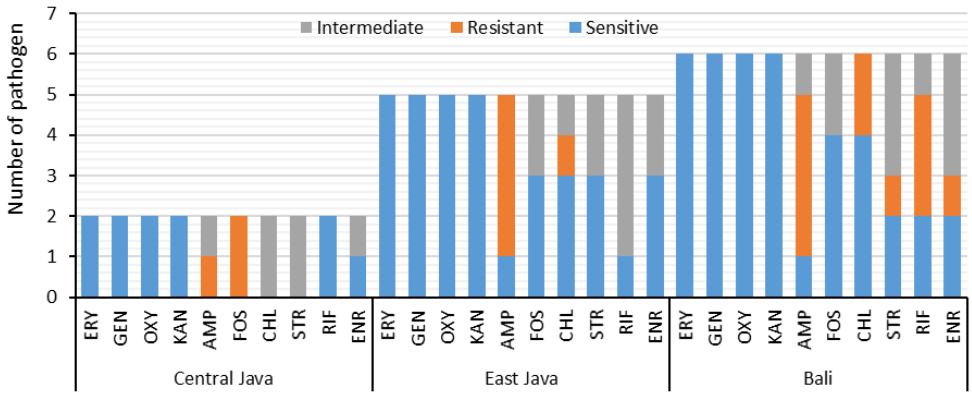

Figure 5. Distribution of antibiotic susceptibility of the pathogenic strains

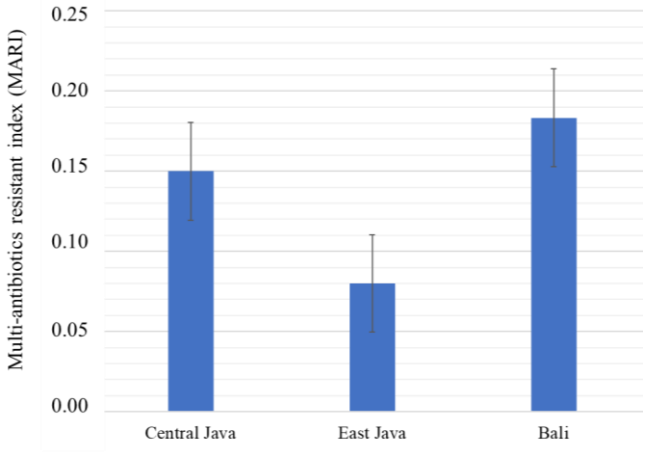

Figure 6. Multi-antibiotic resistance indexes (MARI) in central Java, East Java, and Bali (average ± SE). MARI: number of antibiotics resisted by the bacteria/number of total antibiotics tested (Deng et al. 2020)

Discussion

Disease is an important factor for successful aquaculture. Finfish mariculture in Indonesia is mainly based on grouper and seabass species (La Ode et al. 2015). Hundreds of Vibrio species are found in aquatic environments (Janda 2015). In the present study, we found 13 isolates of pathogenic bacteria from the kidney of diseased *Cantang* Hybrid Grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) with lethargy, skin hemorrhagic, necrosis, fin rot, and mortality in four mariculture area in Indonesia. The aquaculture condition in the fish farms was good based on the water temperature, pH, salinity, dissolved oxygen, and total Vibrio concentrations parameters (Mustafa et al. 2015). The nucleotide sequences of 16S rRNA and DNA gyrase B subunit regions were highly similar to those of *Vibrio harveyi*, *V. alginolyticus*, *V. azureus*, and *Photobacterium damselae subspecies damselae* (previously *V. damsela*) published in the genebank, ranging from 99.66-100% and 98.66-100%, respectively. The results were least different

from previous findings of *V. olivaceus, V. alginolyticus, and V. damsela* from diseased grouper in Karimunjawa Island, Central Java, Indonesia (Sarjito 2011). Ilmiah et al. (2012) reported the association of *V. metchnikovii, V parahaemolyticus*, and *V. mimicus* with moribund grouper collected from the floating net cage in Barru of Sount Celebes, Indonesia. Recently, *V. owenshii* and *V. alginolyticus* with multiple antibiotic resistants were reported as potential pathogenic bacteria from Spermonde Archipelago, Indonesia (Isnansetyo et al. 2022). This evidence confirms that the causative agent of vibriosis is shifting in Indonesian mariculture farms.

This study is the first report on the massive infections of *Photobacterium damselae* subsp. *damselae* in Indonesia marine aquaculture. The pathogen is a member of the Vibrionaceae family, formerly known as *V. damsela* (Smith et al. 1981). This bacterial infection in the present study causes hemorrhagic and necrosis on the skin and fin, apparent bleeding of the peritoneal cavity, swelling kidney, and mortality of the hybrid grouper. These signs are consistent with *P. damselae* subspecies *damselae* infection in damselfish (*Chromis punctipinnis*) (Love et al. 1981), giant grouper (*Epinephelus lanceolatus*) (Jun et al. 2010), seabass (*Dicentrarchus labrax*) (Mahmoud et al. 2018) and other marine farmed fish (Tao et al. 2018). This pathogen has a 16S rRNA gene similar to the *Photobacterium damselae* subsp. *piscicida* but is different phenotypically, especially in motility characteristics, and grows at 37°C (Rivas et al. 2013). There have been no reports of *P. damselae* subsp. *damselae* infection in East Java and Bali marine fish farming until 2016 (Istiqomah et al. 2020). Therefore, the incidence of infection with this pathogen in East Java and Bali in the present study in the period of 2017-2022 fulfills the criteria of an emerging pathogen as the first case found in an area with a major impact (Tengs and Rimstad 2017). Furthermore, the isolation rate (40%) in both locations was higher than those of previous *V. damsela* (14%) in Central Java (Sarjito 2012). That is alarming for fish farmers and stakeholders to take strategic steps to overcome this problem and prevent future unfavorable conditions.

The second emerging pathogen in this study is *V. azureus*. This bacterium was isolated from the kidney of diseased fish in Central Java with a 29% isolation rate. *Vibrio azureus* was first published as a new species isolated from seawater in Japan (Yoshizawa et al. 2009). Species similar to *V. azures* have been reported from waters (Thongchankaew et al. 2011), the digestive tract of Green mussels (*Perna viridis*) (Hikmawati et al. 2019) or associated with sponges (Paul et al. 2021). *Vibrio azureus* has been reported as a potential pathogen causing ulcers in *Holothuria scabra* (Tangestani and Kunzmann 2019). More recently, this bacterial infection was associated with 28% of hemorrhagic cases in Korea's red drum fish (*Sciaenops ocellatus*) farming (Yen et al. 2021). The present study is the first confirmation of *V. azureus* pathogenicity to *Cantang* hybrid grouper in Indonesia. It is therefore suggested that further investigation of the pathogenesis and development of control measures for the disease are important.

Antibiotic resistance has increased in aquaculture (Schar et al. 2021) and aquatic organisms (Hossain and Heo 2022) in the last two decades. We found that 13 disease-causing pathogens in *Cantang* hybrid grouper farming had different resistance patterns to six antibiotics: ampicillin, fosfomycin, chloramphenicol, streptomycin, rifampicin, and enrofloxacin. Even though the spatial multi-antibiotic resistance index (MARI) values of the pathogens in this study were still at a safe level (0.025-0.180). The different resistance patterns of the pathogen in Bali, East Java, and Central Java are susceptible to interactions, along with the free distribution or trading of grouper seeds between provinces in Indonesia (Febriana et al. 2013). Meanwhile, no antibiotic resistance observations were made from Batam since no pathogenic bacteria were found in the area in the present study.

Vibrio harveyi and *V. alginolyticus* are Indonesia's most frequently reported pathogens in grouper aquaculture, although the inactivated vaccines for this *Vibrio* spp were commercially available and used by fish farmers (Istiqomah et al. 2020). Both groups of pathogens still appear in the present surveillance study in Bali with a spatial multi-antibiotic resistance index of 0.180 (greater than other areas). Although the value is close to the safe threshold of 0.2 (Deng et al. 2020), more proper biosecurity steps, such as fish screening and quarantine, are needed to avoid introducing and spreading these pathogens in aquaculture facilities.

It is essential to strengthen antibiotic use control, enhance diagnostic services for the fish farmer, and keep excellent water quality and hygiene practices, along with the improvement of feeding management (Apines-Amar et al. 2012), selective breeding (Yang et al. 2020) or development of other control measures, such as biocontrol or probiotics (Li et al. 2019), enhancement of the host immune by the revitalization of vaccine (Pang et al. 2020) or application of immunostimulant (Kuo et al. 2020). These efforts will prevent horizontal resistant gene transfer from aquaculture to human pathogens (Shen et al. 2019) and prevent the occurrence of multiple antibiotic-resistant strains (MARI>0.2), as has been reported in tilapia (*Oreochromis* sp.) (Arumugam et al. 2017), white-leg shrimp (*Litopenaeus vannamei*) (De Silva et al. 2018), and other farmed or wild-caught aquatic animals (Schar et al. 2021).

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