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Characterization and molecular detection of pathogenicity and antibiotic-resistance genes in *Vibrio parahaemolyticus* isolated from Pacific white shrimp

Karakterisisasi dan deteksi molekular gen penyandi patogenitas, resistansi antibiotik pada *Vibrio parahaemolyticus* yang diisolasi dari udang vanamei

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ABSTRACT

This study aimed to conduct the characterization and molecular detection of the pathogenicity and antibiotic-resistance genes in *Vibrio parahaemolyticus*, as the causative agent of vibriosis in Pacific white shrimp. The *V. parahaemolyticus* isolates were collected from the shrimp's hepatopancreas, before biochemical test and polymerase chain reaction (PCR) sequencing of the 16S rRNA gene confirmation. The hemolysis test and PCR were applied to detect the presence of virulence genes, namely *tox*R, thermostable direct haemolysin (tdh), and tdh-related haemolysin (trh). The Kirby-Bauer method was used for characterizing the resistance patterns against ampicillin (AMP), tetracycline (TET), cyprofloxacin (CIP), enrofloxacin (ENR), and chloramphenicol (CHL). The biochemical tests and PCR-16SrRNA gene sequencing confirmed that 12 isolates belonged to *V. parahaemolyticus* that were further verified by amplification of the *tox*R gene in 382 bp (100% of the isolates). The alpha hemolysis activity was also confirmed by the amplicon of 199 bp in all isolates. All *V. parahaemolyticus* isolates showed their resistance to AMP and 42% of the isolates were TET-resistant. However, no resistance was shown to CIP, ENR, and CHL. The PCR-based analysis resulted a detectable resistance gene of *amp*C (42% of the isolates) and *tet*B (83% of the isolates).

Keywords: antibiotics, shrimp, resistance, virulency, *Vibrio parahaemolyticus*

ABSTRAK

Penelitian ini bertujuan untuk melakukan karakterisasi dan deteksi molekular dari gen patogenisitas dan resistansi antibiotik pada *Vibrio parahaemolyticus*, agen penyebab vibriosis pada udang vaname. Isolat *V. parahaemolyticus* dikoleksi dari hepatopankreas, diuji secara biokimiawi dan selanjutnya dikonfirmasi dengan polymerase chain reaction (PCR)-sekuensing dari gen 16S rRNA. Tes hemolisis dan metode PCR diterapkan untuk mendeteksi keberadaan gen virulensi *tox*R, thermostable direct haemolysin (tdh) and tdh-related haemolysin (trh). Metode Kirby Bauer digunakan untuk karakterisasi pola resistansi terhadap ampisilin (AMP), tetrasiklin (TET), kloramfenikol (CHL), siprofloksasin (CIP) dan enrofloksasin (ENR). Uji biokimia dan sekuensing gen PCR-16SrRNA memastikan bahwa 12 isolat adalah *V. parahaemolyticus* yang selanjutnya diverifikasi dengan amplifikasi gen *tox*R berukuran 382 bp (100% isolat). Aktivitas alfa hemolisis juga dikonfirmasi dengan amplikon PCR (199 bp) di semua isolat. Seluruh isolat *V. parahaemolyticus* menunjukkan resistansinya terhadap AMP, 42% resistan TET, tidak ada resistansi yang ditunjukkan pada CIP, ENR dan CHL. Analisis berbasis PCR menghasilkan gen resistan yang terdeteksi dari gen *amp*C (42% isolat) dan gen *tet*B (83% isolat).

Kata kunci: Antibiotik, udang, resistansi, virulensi, *Vibrio parahaemolyticus*

INTRODUCTION

Vibrio spp. bacteria can be found in the marine and estuary environments around the world (Rocha *et al*., 2016). These bacteria can also live inside the fish and aquatic crustacea body. These genera exist as parts of normal microflora (Rocha *et al*., 2016). Several *Vibrio* spp., such as *Vibrio anguilarum*, *V. harveyi*, and *V. parahaemolyticus* can cause vibriosis in shrimps (Brober *et al*., 2011). Currently, there is a disease called acute hepatopancreatic necrosis disease (AHPND) or early mortal syndrome (EMS). EMS has caused a significant loss for shrimp production in Southeast Asia countries (Flegel, 2012; Leano and Mohan, 2012; NACA-FAO, 2014) and Mexico (Nunan *et al*., 2014). This disease is caused by *V. parahaemolyticus* and affects the hepatopancreas activity of the infected shrimps (Tran *et al*., 2013). AHPND causes a mortality up to 100% both in Pacific white shrimp and tiger prawn (Zorriehzahra and Banaederakhshan, 2015).

Unfortunately, not all strains of *V. parahaemolyticus* are pathogenic. The bacterial pathogenicity is closely related to the bacterial capability in haemolysis process. The thermostable direct haemolysin (tdh) and TDH-related haemolysisn (trh) are two main virulence factors of *V. parahaemolyticus* during the infection process. The tdh and trh genes are responsible for haemocyte membrane damage or haemolysis process (Raghunath 2015).

One of the effective controls required to prevent the disease caused by vibrio bacteria is the use of antibiotics. Antibiotics have been misused for preventive and disease treatments, even as a growth promoter for shrimps (Rodrigues *et al*., 2011). The uncontrollable and continous use of antibiotics in shrimp culture will impact on the resistant vibrio strain development (Rocha *et al*., 2016; Rodrigues *et al*., 2011). Kusmawarti *et al*. (2017) reported 36 isolates of *V. parahaemolyticus* originated from shrimp pond in West Java, Central Java, and East Java were tested their resistance level against eight antiobiotics (doxycycline, nitrofurantoin, cyprofloxacin, nalidixic acid, amoxicillin-clavulanic acid, chloramphenicol, streptomycin, and erythromycin) and resulted in 100% isolates were resistant to streptomycin, 90% isolates were resistant to erythromycin, and the total resistant isolates against amoxicillin-clavulanic acid and nitrofurantoin were 83.33% and 58.33%, respectively.

Molecular technical approach studies with PCR method have many been performed to identify the resistance pattern related to the resistant-encoded genes against antibiotics in bacteria (Momtaz *et al*. 2012). Several bacteria discovered can naturally survive against more than one antibiotic agent. The resistance characteristics are not only obtained from genetic mutation, but also horizontal gene transfer from the DNA of other bacteria. This study aimed to conduct a pathogenicity molecula characterization, determine the resistance category (sensitive, intermediate, and resistant), and detect the antibiotic-resistant encoded genes of *V. parahaemolyticus* as vibriosis causative agent in shrimp.

MATERIALS AND METHODS

Bacterial isolates and characterization *V. parahaemolyticus*

The *V. parahaemolyticus* bacteria were the samples isolated from the shrimp culture centers. The bacterial isolation and identification were performed following the Indonesian National Standard (SNI) 01-2332.5-2006 (BSN, 2006) with several modifications. The *V. parahaemolyticus* bacteria were grown on the thiosulfate citrate bile sucrose agar (TCBSA) media, before being streaked quadrantly on trypticase soy agar (TSA) added with 3% NaCl. The single bacterial colony was the grown on the TSA slant and preserved at 4°C as a stock culture.

The *V. parahaemolyticus* bacteria were verified conventionally with Gram staining, catalase test, oxidase test, gelatinase test, and haemolysis test, followed by biochemical test using *API KIT 20E*. Furthermore, molecular confirmation with PCR method was performed using the 16S rRNA encoded gene primers, namely 27F 5′- AGA GTT TGA TCM TGG CTC AG -3′ and 1492R 5′- CGG TTA CCT TGT TAC GAC TT-3′ (Hasan *et al.,* 2017).

V. parahaemolyticus pathogenicity test

The pathogenicity test was performed on blood agar media. The media color changes were observed after the isolate was cultured for 24 hours to determine its haemolysis type. Molecular pathogenicity test was performed by detecting the specific virulence-encoded genes of *V. parahaemolyticus*, such as *toxR,* thermostable direct haemolysin (tdh), and tdh-related haemolysin (trh) with PCR method (Table 1).

Resistance pattern against antibiotics

Antibiotic resistance test was performed using the Kirby-Bauer disk diffusion test on a Mueller-Hinton agar as a culture medium based on the Clinical and Laboratory Standards Institute Guidelines (CLSI, 2016). The antibiotics used in this study were 10 µg amphicillin (AMP), 30 µg tetracycline (TET), 30 µg chloramphenicol (CHL), 5 µg ciprofloxacin (CIP), and 5 µg enrofloxacin (ENR). The bacterial suspension and colonies were diluted to reach the 0.5 McFarland standard (equivalent to 1.5×10^8 CFU/ml), then 1 ml of the suspension was spread around the Mueller-Hinton agar. The disk papers containing antiobiotics were placed on the Mueller-Hinton agar medium using a sterile pinset with similar distance and incubated at 37°C for 18-24 hours, before being counted their clear zone based on CLSI (2016).

***V. parahaemolyticus* DNA extraction**

Extraction was performed on *V. parahaemolyticus* bacteria with intermediate to highly antibiotic resistant category. The boiling method was used for DNA extraction by mixing the bacterial colony obtained from the blood agar culture into the 1.5 ml microtube filled with 1 ml PBS, then homogenized with a vortex. This suspension was centrifuged at 10000 rpm for 3 minutes to precipitate the bacterial cells as a pellet, which was taken at 100 µl into a 1.5 ml microtube. The 200 µl nuclease-free water was added into the microtube and homogenized with a vortex, before incubation at 95°C for 10 minutes. The mixture was centrifuged at 10000 rpm for 3 minutes. The supernatant as DNA was taken at 100 µl for resistant-encoded gene detection with PCR (Qabajah *et al*., 2014).

Antibiotic-resistant encoded gene detection

The antibiotic-resistant encoded genes were detected using *ampC* (amphicillin), *tetA*(A), and *tetB*(B) (tetracycline) primers with PCR (Table 2). The total PCR reaction sample was at 10 µl, containing 5 µl mastermix, 1.2 µl primer, 3.8 µl H_2O, and 1 µl DNA genom. The amplification process included 35 cycles starting from pre-denaturation at 95°C for 3 minutes, then amplification process with denaturation at 95°C for 30 seconds, annealing at 50-60°C for 30 seconds, extension at 72°C for a minute, and final extension at 72°C for 5 minutes. The amplified DNA sample was visualized by running it on an agarose-gel electrophoresis.

Table 1. Specific virulence-encoded gene primers of *tox R,* tdh*,* trh in *V. parahaemolyticus.*

Primer	Target gene	Sequence (5'-3')	Fragment size (bp)	Reference
*tox*R-F	*toxR*	GAAGCAGCACTCACCGAT	382	Chen & Ge, 2010
*tox*R-R	*toxR*	GGTGAAGACTCATCAGCA	382	Chen & Ge, 2010
tdh-F	Tdh	CCACTACCACTCTCATATGC	199	Li *et al*., 2016
tdh -R	Tdh	GGTCTAAATGGCTGACATC	199	Li *et al*., 2016
trh -F	Trh	GGCTCAAAATGGTTAAGCG	250	Li *et al*., 2016
trh -R	Trh	CATTTCCGCTCTCATATGC	250	Li *et al*., 2016

Table 2. The AMP and TET antiobiotic-resistant encoded genes used in the study.

Antibiotics	Target gene	Primer sequence	Fragment size (bp)	Annealing temperature
AMP[a]	*amp*C	(F) 5′-AATGGGTTTTCTACGGTCTG-3′ (R) 5′-GGGCAGCAAATGTGGAGCAA-3′	191	55°C
TET[b]	*tet* (A)	(F) 5′-GGTTCACTCGAACGACGTCA-3′ (R) 5′-CTGTCCGACAAGTTGCATGA-3′	577	56°C
	tet (B)	(F) 5′-CCTCAGCTTCTCAACGCGTG-3′ (R) 5′-GCACCTTGCTCATGACTCTT-3′	634	56°C

Note: [a]AMP: Amphicillin (Jung *et al*., 2021), [b]TET: Tetracycline (Belaynehe *et al*., 2018)

RESULTS AND DISCUSSIONS

Results

Bacterial sample culture and identification

The 12 isolates of *V. parahaemolyticus* were successfully isolated from the shrimps. The isolates grew well on thiosulphate citrate bile salt sucrose (TCBS) media with 3% NaCl addition. Based on the macroscopic observation, the colony formed had a green color with 3-5 mm diameter, small-to-medium round shape, convex elevation, and smooth surface. The single colony of *V. parahaemolyticus* that grew on blood agar had a circular shape with smooth surface, convex elevation, and white opaque color. After Gram staining, the 12 isolates microscopically showed the morphology of *V. parahaemolyticus*, namely short-rod shape and pink cell color (Gram negative).

V. parahaemolyticus identification with biochemical test and API KIT 20E

The *V. parahaemolyticus* pathogenicity test results performed on blood agar showed that the bacteria could lyse the blood cells, visualized as alpha-haemolysis, whereas the clear zone was formed around the streaking region of the grown bacteria on a clearer media, but non-transparent. The formation of lysis zone on blood agar indicates that the isolate can lyse red blood cells. The alpha-haemolysis is related to the partial red blood cell and haemoglobin lysis (Buxton, 2013). Based on the serological test and *API KIT 20E*, the isolates were significant to *V. parahaemolyticus* with an excellent identification accuracy index at 99.9% (Table 3).

V. parahaemolyticus molecular identification and specific 16S rRNA gene detection with PCR

The 12 positive isolates based on the biochemical test were molecularly identified by determining the available 16S rRNA. The molecular identification results of the isolates showed that the 12 isolates positively had the 16S rRNA with 1450 bp amplicon (Figure 1).

Table 3. Biochemical identification test with KIT API 20E on *V. Parahaemolyticus* isolate.

Physiological test	Reaction	Physiological test	Reaction
ONPG (ortho-Nitrophenyl-β-galactopyranoside)	-	Citrate	+
ODC (Ornithine decarboxylase)	-	Glucose	+
LDC (Lysine decarboxylase)	+	Manose	+
ADH (Arginine Dihydrolase)	+	Inositole	-
TDA (Tryptophan deaminase)	-	Melobiose	-
H_2S	-	Sorbitole	-
Urease	-	Rhamnose	-
Indol	-	Sucrose	-
VP (Voges-Proskauer)	+	Arabinose	-
Gelatin	-	Oxidase	+

Note: + = positive reaction; - = negative reaction

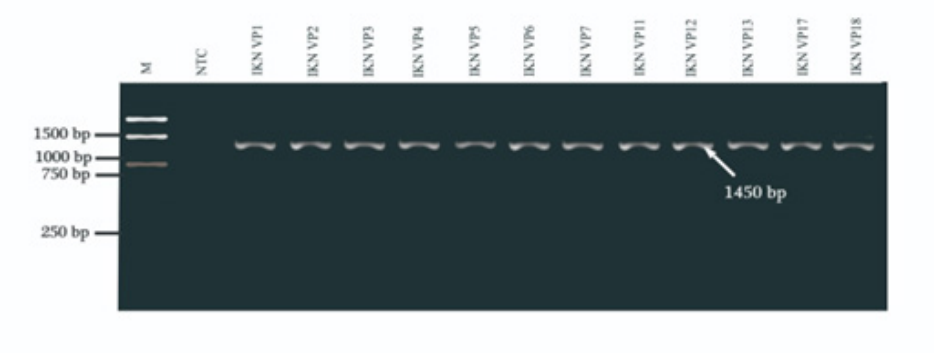

Figure 1. The amplification of 16S rRNA gene (1450 bp) in *V. parahaemolyticus*.The 12 isolates showed an amplification of 16S rRNA gene. M: marker 100 bp; NTC: non template control.

The ribosomal RNA (rRNA) gene is the most conserved gene. This condition causes this gene to be used as a universal primer, which can be determined its nucleotide sequence through sequencing (Letchumanan *et al*., 2015). The sequence portions of rDNA from each species that are genetically correlated, are commonly similar. Therefore, each organism that has a certain kinship can be equivalent and easier to distinguish based on the sequence as the main characters of the organism. The PCR product sequencing phase was performed on four representative isolates with varying resistance, namely resistant, intermediate and sensitive to the TET antibiotics (Table 6), namely IKNVP1, IKN VP3, IKN VP4 and IKN VP11 isolates. This 16S rRNA gene sequencing was performed to identify the nucleotide base sequence of the isolate samples and the isolate identification results based on the nucleotide BLAST (Basic Local Alignment Search Tool from NCBI) analysis in the GenBak (Figure 2). The DNA sequence is an important information to determine the identity, function, and modification of DNA fragment or gene.

The DNA sequencing analysis results on the sample isolates using BLAST analysis indicates that IKN VP1 and IKN VP4 isolates have 100% similarity level to *V. parahaemolyticus* strain 2012V-1165 and IKN VP11 isolate has 99% similarity level to the following bacterial species. For IKN VP3 isolate, it is homologous or similar to *V. parahaemolyticus* strain TY-41 at 99% (Table 4). According to Singh *et al*. (2011), BLAST pairwise alignment can identify the homologous sequence part, that demonstrates the structural, functional, and evolution relationships between

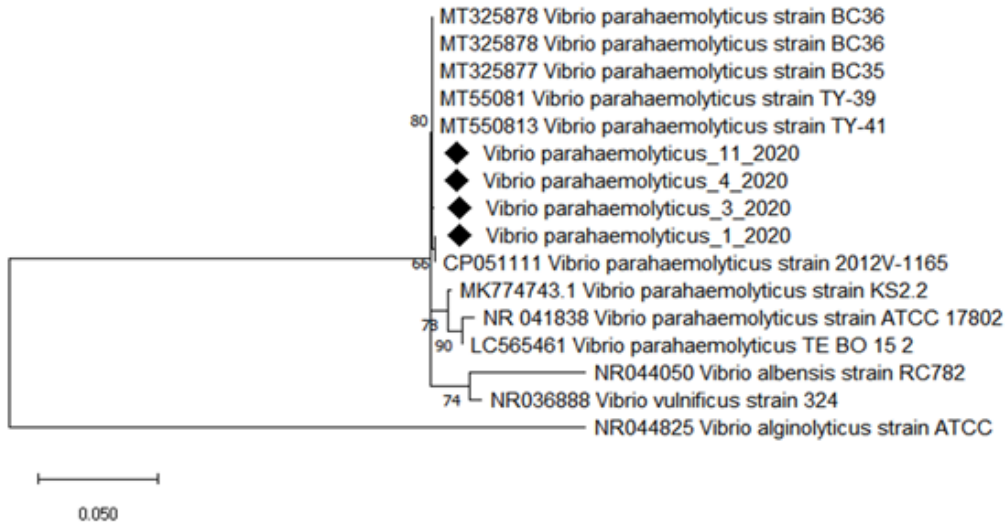

Figure 2. The phylogenetic tree based on the sequence data information of 16S rRNA gene.

Table 4. The nucleotide BLAST analysis of 16S rRNA fragment (1450 bp) on four isolates.

Sample	*Closed-relative*	MS	TS	QC (%)	EV	ID (%)	ACC
IKN VP1	*Vibrio parahaemolyticus strain 2012V-1165 chromosome 1*	2627	38676	98	0.0	100	CP 051111.1
IKN VP3	*Vibrio parahaemolyticus strain TY-41 16S ribosomal RNA gene*	1557	1557	79	0.0	99	MS 550813.1
IKN VP4	*Vibrio parahaemolyticus strain 2012V-1165 chromosome*	2628	38753	98	0.0	100	CP 051111.1
IKN VP11	*Vibrio parahaemolyticus strain 2012V-1165 chromosome*	2599	38310	98	0.0	99	CP 051111.1

Note: MS = Max Score, TS = Total Score, QC = Query coverage, EV = Expected Value, ID = Identity, ACC = Accession

both DNA sequences. The phylogenetic tree is actually used to compare the equivalent genes originated from several species to reconstruct the kinship among the species, which represents the similarity among species. The purpose of phylogeny is to reconstruct the history of life and explain the diversity of living things (Claverie & Notredame, 2007).

The molecular identification results with PCR showed that the 12 isolates were amplified to have a specific *toxR* gene at 382 bp amplicon (Figure 3). The *toxR* gene controls the enzymatic functions, that have roles in toxin release, either endotoxin or exotoxin (Chen & Ge, 2010).

The PCR amplification of virulence genes, namely toxin-encoded gene, *toxR*, and hemolysin-encoded genes, thermostable direct haemolysin (tdh) and tdh-related haemolysin (trh), indicates that all 12 isolates of *V. parahaemolyticus* have specific *toxR* gene at 382 bp amplicon (Figure 3) and tdh gene as hemolysin-encoded gene, detected and amplified at 199 bp (Figure 4). Nevertheless, another virulence gene, i.e. trh, indicates a negative result (unamplified) (Table 5).

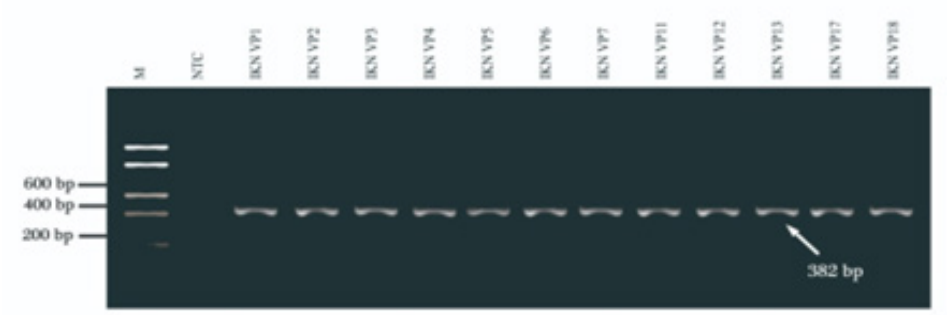

Figure 3. The PCR product visualization of *toxR* gene (382 bp) on *V. parahaemolyticus.* The 12 isolates indicate an amplification of *toxR* gene at 382 bp molecular weight. M: marker 100 bp; NTC: non template control.

Table 5. The PCR amplification of virulence genes (*toxR,* tdh, and trh) on 12 isolates of *V. parahaemolyticus.*

		Virulence genes		
Isolate Code	Origin	*toxR*	tdh	trh
IKN VP1	Karawang	+	+	-
IKN VP2	Karawang	+	+	-
IKN VP3	Karawang	+	+	-
IKN VP4	Karawang	+	+	-
IKNVP5	Karawang	+	+	-
IKN VP6	Karawang	+	+	-
IKN VP7	Serang	+	+	-
IKNVP11	Pangandaran	+	+	-
IKN VP12	Pangandaran	+	+	-
IKN VP13	Anyer	+	+	-
IKN VP17	Indramayu	+	+	-
IKN VP18	Indramayu	+	+	-

Note: + = Amplified; - = Unamplified

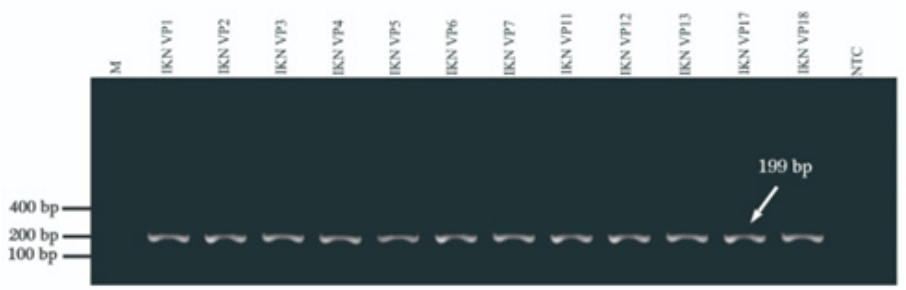

Figure 4. The amplification of tdh gene (199 bp) on *V. parahaemolyticus.* All 12 isolates positively have tdh at 199 bp. M: marker 100 bp; NTC: non template control.

Not all *V. parahaemolyticus* strains are correlated with the shrimp infectious disease and mortality, but the virulence factors are generally related to the available virulence factor expression, such as thermostable direct hemolysin (tdh) and thermostable direct hemolysin related hemolysin (trh) (Broberg *et al*., 2011; Raghunath 2015).

Antibiotic sensitivity (β-lactams, tetracyclines, quinolones, and choramphenicols) with disk diffusion method

The antibiotic sensitivity test results of 12 samples based on the inhibition zone formation on Mueller-Hinton agar showed 100% resistance level against amphicillin, followed by 42% resistance level against tetracycline (5 of 12 isolates) (Table 6). Moreover, the antibiotic sensitivity test results also showed the formation of inhibition zone categorized as intermediate resistance level, namely against tetracycline (16%), ciprofloxacin (75%), enrofloxacin (42%), and chloramphenicol (17%). The sensitive category from 12 isolates showed that 42% of isolates were sensitive against tetracycline, 83% were sensitive against chloramphenicol, 58% were against enrofloxacin, and 25% were against ciprofloxacin.

Several isolates showed a multi-resistance condition against two or more antibiotics. The most frequent multi-resistance condition was found against the β-lactam group (amphicillin) and tetracycline group (Table 6 and 7). The five isolates (42%) in this study were multi-resistant based on the sensitivity test using disk diffusion method. Meanwhile, the 58% isolates (7 of 12 isolates) were resistant against one antibiotic type (amphicillin) (Table 6 and 7).

Antibiotic-resistance encoded gene detection

The antibiotic-resistance encoded gene detection was performed molecularly on *V. parahaemolyticus* isolate. Table 7 presents the detection results of *ampC*, *tet(A)*, and *tet(B)* genes. The *ampC* gene detection was positively presented on five isolates (42%) that were resistant against

Table 6. Antibiotic resistance pattern of pathogenic *V. Parahaemolyticus* isolates.

No.	ISOLATE	AMP[a]	TET[b]	CHL[c]	ENR[d]	CIP[e]
1	IKN VP1	R	R	S	S	S
2	IKN VP2	R	R	S	S	S
3	IKN VP3	R	I	S	S	I
4	IKN VP4	R	S	S	S	I
5	IKN VP5	R	S	I	I	I
6	IKN VP6	R	S	S	I	I
7	IKN VP7	R	I	I	I	I
8	IKN VP11	R	R	S	S	I
9	IKN VP 12	R	S	S	I	I
10	IKN VP 13	R	S	S	S	I
11	IKN VP17	R	R	S	S	I
12	IKN VP18	R	R	S	I	S

[a]AMP: Amphicillin, [b]TET: Tetracycline, [c]CHL: Chloramphenicol, [d]EN: Enrofloxacin, [e]CIP: Ciprofloxacin, R: resistant, I: Intermediate, S: Sensitive.

Table 7. Antibiotic resistance on *V. parahaemolyticus* (n=12).

	Resistance category		
Antibiotics	Sensitive	Intermediate	Resistant
Amphicillin	0%	0%	100%
Tetracycline	42%	16%	42%
Chloramphenicol	83%	17%	0%
Enrofloxacin	58%	42%	0%
Ciprofloxacin	25%	75%	0%

amphicillin at 191 bp amplicon (Figure 5). The antibiotic resistance can occur due to available resistance gene expression.

Genotypically, the *ampC* gene amplicon could be detected in five isolates, namely IKNVP1, IKNVP7, IKNVP11, IKNVP12, and IKNVP18 or about 42% of all observed isolates, although disk diffusion method phenotypically showed that 12 isolates of *V. parahaemolyticus* obtained resistance pattern against amphicillin antibiotic. The *tet(A)* and *tet(B)* resistance genes in 12 isolates that were resistant against tetracycline group obtained 83% of isolates were positively amplified *tet(B)* (Figure 6) at 634 bp amplicon, while no isolates detected *tet(A)* gene. In this study, the *tet(B)* gene was more dominant than the *tet(A)* gene.

Discussions

Vibriosis disease caused by pathogenic *Vibrio* sp. still becomes a problem in Pacific white shrimp culture. In this study, the sample isolates could lyse the red blood cells and produce α-haemolysis activity in blood agar. The clear zone formation on blood agar indicates that the isolates can lyse red blood cells. The α-haemolysis phenotype in a zone around the colony or streaking area is observed as a partial lysis of red blood cells and haemoglobins (Buxton, 2013). Disease incident is often closely associated with bacterial pathogenicity, tissue invasion capability, colonization, proliferation speed, besides the host immunity against bacteria (Tortora *et al*., 2016). Haemolysis activity causes the bacteria to attack the host defense by lysing the blood cells. The living bacteria will enter the bloodstream to the target organ and systemically spread to whole host body (Fitriatin & Manan, 2015).

The *V. parahaemolyticus* bacteria can ferment glucose and mannose, but seems unable to ferment sorbitole, arabinose, rhamnose. The lysin decarboxylase, arginin dihydrolase, and Voges-Proskauer (VP) tests showed positive results. Meanwhile, ornithin decarboxylase, H_2S

Table 8. Resistance level based on antibiotic groups and resistance encoded genes (n=12).

Positive amplification of resistance gene				
Antibiotic group	Total resistant Isolates	Resistance gene	Total isolates	Percentage
β-lactam	12	*ampC*	5	42%
Tetracyclines	5	*tet(A)*	0	0%
		tet(B)	10	83%

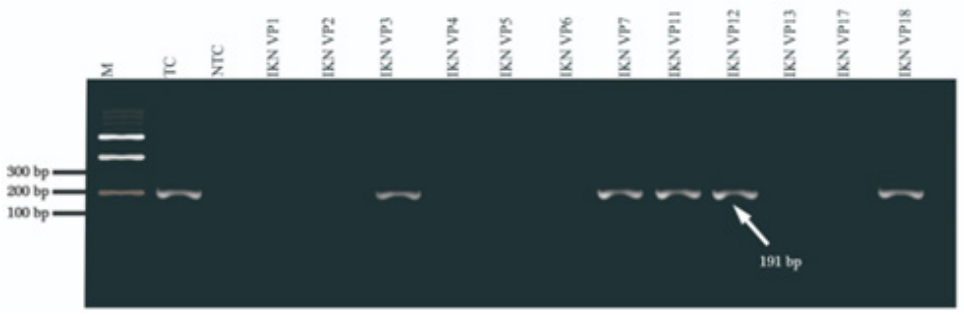

Figure 5. The PCR amplification of *ampC* (191bp) on *V. parahaemolyticus*. Five isolates indicate positive results of *ampC*. M: marker 100 bp; NTC: non template control.

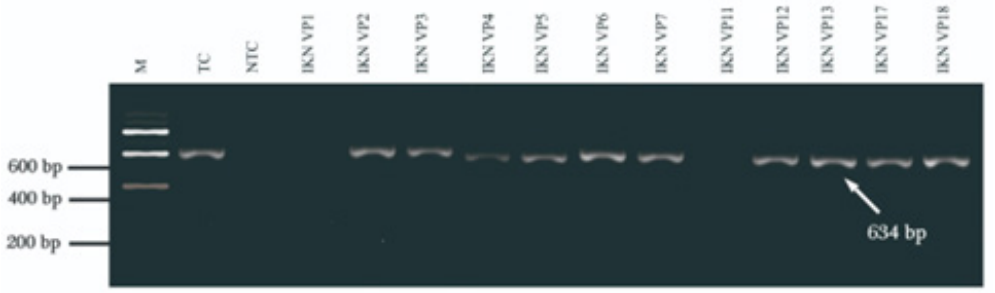

Figure 6. Amplification of *tet(B)* gene (634 bp) as a tetracyline-resistance encoded gene in *V. parahaemolyticus.* Ten isolates indicate positive results of *tet(B)* gene. M: marker 100 bp; NTC: non template control.

production, urease, gelatin, indole, and tryptophan deaminase tests showed negative results.

The molecular genotypic identification results with PCR showed that the 12 sample isolates were amplified to have *toxR* specific gene at 382 bp amplicon size (Figure 2). Chen & Ge (2010) stated that the *toxR* gene could control several enzyme functions, which had roles in toxin release process, either endotoxin or exotoxin. In this study, there was a bacterial isolate detected to have more than one virulence encoded gene. From the 12 sample isolates, 100% isolates were amplified to have two virulence genes, namely *toxR* gene (382 bp) and tdh gene (199 bp). Not all *V. parahaemolyticus* strains cause disease, but the ones that cause disease are commonly related to the available virulence factors, namely thermostable direct hemolysin (tdh) or thermostable direct hemolysin related hemolysin (trh) (Broberg *et al.,* 2011; N). Although all samples were detected to have *toxR* and tdh genes, this condition was contradictory with another virulence gene, namely the trh gene (tdh-related haemolysin). The trh gene detection showed a negative result (unamplified). This was thought due to the incapability of primers attaching the incomplementary trh encoded DNA; trh gene is non-conservative gene, which causes a mismatching with the available primers. Also, these 12 isolates were actually thought to have no trh gene.

These isolate virulences are still necessary to testify further against shrimps. The *V. parahaemolyticus* sample virulence may be more controlled by the tdh gene expression. *V. parahaemolyticus* has two virulence factors, namely porous-forming protein called tdh, which contributes in disease transmission, and the undetected trh. The trh is an encoded gene to cause infection/disease pathogenicity mechanism (Letchumanan *et al*., 2015). Various available virulence genes variability that can be amplified may also because of *Vibrio* sp. bacteria group characteristics as opportunistic pathogen bacteria. These bacteria are saprophytic and can turn into pathogenic in a certain environment and abundance (Kadriah *et al*., 2013).

Several isolates showed a multi-resistance condition against two or more than three antibiotics. The most multi-resistant incidence was observed on the β-lactam (amphicillin) and tetracycline groups (Table 7 and 8). Multiple drugs resistance (MDR) are defined as a resistance against two or more drugs, even drug-classified materials (Walsh 2003). Five isolates (42%) in this study were multi-resistant based on the sensitivity test with the disk diffusion method. Meanwhile, 58% isolates (7 of 12 isolates) were resistant against one antibiotic type (amphicillin) (Table 7 and 8). Based on the Indonesian Minister of Marine and Fisheries Affairs Regulation of 1/ Permen-KP/2019 about fish drugs, antibiotics are included as hard drugs, as the permittable groups for limited usage are tetracycline, macrolide, fluoroquinolone, and sulfonamide groups. The emergence of resistant isolates against β-lactam (amphicillin) and tetracycline antibiotics in this study becomes an important information, due to suspected as a transfer of resistance encoded genes among bacteria in the aquaculture environments.

Antibiotics are used as a handling (medication) effort against the disease or growth promoter for shrimps (Rodrigues *et al*., 2011). The uncontrollable use of antibiotics in shrimp culture can impact on the multi-resistant development of *Vibrio* sp. strains (Rocha *et al*., 2016; Rodrigues *et al*., 2011). Kusmawarti *et al*. (2017) suspected that several types of antibiotics routinely applied by commercial shrimp hatcheries in Indonesia could cause the emergence of antibiotic-resistant vibrio strains.

The ampicillin resistance distribution in the *V. parahaemolyticus* sample was thought due to vertical or horizontal gene transfer with other bacteria, especially through the R-plasmid (Molina-Aja *et al*., 2002), but this needs a further investigation. The use of oxytetracycline antibiotics as a growth promoter is thought as a cause of high tetracycline resistance incident (Molina-Aja *et al*., 2002). The antibiotic growth promoter (AGP) is the use of antibiotics under therapeutic doses, which are inoptimally absorbed by body cells and can stimulate the antibiotic resistance. Reda *et al*. (2013) explained that the misuse of antibiotics in fish feed as growth promoters is more frequent than as disease control. The negative impact of antibiotics as growth promoters in aquaculture activities can cause pathogenic bacteria and normal flora bacteria elimination, which decreases the loss risk due to bacterial disease and increases the shrimp production. In this study, the isolates were resistant against β-lactam (100%) and tetracycline (42%) antibiotics (Tables 6 and 7). These antibiotics are no longer used in aquaculture as they have been banned since before 2010, along with the regulation of PERMEN KKP no 1 about fish drugs. However, the bacterial isolates from

aquaculture products are often currently found resistant to antibiotics (Kusmarwati *et al*., 2017), due to gene transfer processes that occur vertically or horizontally in waters and sediments, as well as microenvironments such as in fish and shrimp intestines (Molina-Aja *et al*., 2002).

In this study, the 10 bacterial isolates were resistant against two antibiotics (amphicillin and tetracycline) of five antibiotics observed (Tables 8 and 9). All *V. parahaemolyticus* isolates from various locations were resistant against amphicillin (100%), followed by tetracycline (42%). The existence of resistant bacterial strains against several antibiotics indicates that there has been a vertical or horizontal transfer of resistant encoded genes among bacteria in the environment, which are often exposed to excessive antibiotics dose, either from aquaculture or agriculture media (Letchumanan *et al*., 2015). Most isolates were still sensitive against chloramphenicol and enrofloxacin (Tables 6 and 7). Low resistance on chloramphenicol and enrofloxacin in this study was thought due to the limited use of these antibiotics in excess doses. This result was similar to Letchumanan *et al*. (2015), who reported that 98% of *V. parahaemolyticus* isolates were sensitive to imipenem, amphicillin sulbactam (96%), chloramphenicol (95%), and gentamycin (85%). According to Suwiryono (2017), the *V. parahaemolyticus* isolate sensitivity to quinolone antibiotics such as enrofloxacin is still high. This means that enrofloxacin is an effective *in-vitro* antibiotic.

Bacterial resistance against amphicillin antibiotic is related to the existence of *ampC* gene in the cell (Kurnia *et al*., 2018). Genotypically, *AmpC* gene was found on five isolates, namely IKNVP1, IKN VP7, IKNVP11, IKNVP12, and IKNVP18 or about 42% of the observed isolates. according to Liu and Liu (2016), the β-lactamase *ampC* enzyme can be encoded by many genes, such as *bla*$_{ACC}$, *bla*$_{CMY}$, *bla*$_{MOX}$, *bla*$_{DHA}$, *bla*$_{CIT}$, and *bla*$_{EBM}$. Therefore, this study results cannot be classified as a false-positive due to other mechanisms that can cause amphicillin-resistance condition. A horizontal resistant-gene transfer has many been occurred, either in aquatic and terrestrial environments, which can be a key effect of amphicillin-resistance characteristics in the nature (Letchumanan *et al*., 2015). The resistance-encoded gene detection of *tet(A)* and *tet(B)* on 12 resistant isolates against tetracycline obtained 83% isolates were positive to have *tet(B)* at 634 bp amplicon and no amplified isolates had a positive result on *tet(A)* (Figure 14). In this study, the *tet(B)* gene indicates to be more dominant than the *tet(A)* gene. In addition to *tet(A)* and *tet(B)* genes, other genes contributed to tetracycline resistance are *tet(C)*, *tet(D)*, and *tet(E)* (Zhu *et al*., 2018).

CONCLUSION

All isolates investigated in this study are *Vibrio parahaemolyticus*. The detected virulence-encoded genes, namely *toxR* (100%) and tdh (100%), were closely related to the pathogenic characteristics on shrimps. All *V. parahaemolyticus* isolates obtained in this study were resistant to amphicillin (100%), 42% isolates were resistant to tetracycline. There were no *V. parahaemolyticus* isolates resistant to quinolones and chloramphenicols in this study. The resistant-encoded genes that could be detected were *ampC* at 42% and *tet* at 83%.

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