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Detection of Bacteriophages Specific to *Vibrio parahaemolyticus* in Marine Water Samples, Surat Thani, Thailand

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Abstract

Vibrio parahaemolyticus is a halophilic bacterium commonly found in estuarine and marine water. It is a seafood-borne pathogen that causes acute gastroenteritis worldwide. *V. parahaemolyticus* bacteriophage is a virus that infects *V. parahaemolyticus* bacteria. The application of a bacteriophage that can eliminate pathogenic bacteria may decrease the severity of outbreaks. The objectives of this study were to determine water quality and isolate *V. parahaemolyticus* bacteriophage from various marine water environments. Ten marine water samples from Surat Thani province were examined, consisting of shrimp pond water (6 samples), estuarine water (1 sample), and seawater (3 samples). Each water sample was tested for physical quality using environmental parameters. Results revealed that temperature, pH, dissolved oxygen (DO), oxidation-reduction potential (ORP), and salinity were in the ranges of 25.6°C-30.6°C, 7.26-8.64, 0.00-4.45 mg/L, (-)30-181 mV, and 1.0-30 ppt, respectively. Total viable bacterial counts on plate count agar (PCA) were in the ranges of $1.2x10^3$-$7.2x10^3$ CFU/ml, $1.2x10^4$ CFU/ml, and $1.8x10^6$-$2.5x10^6$ CFU/ml for the six shrimp pond water, one estuary, and three seawater samples, respectively. Green colonies on Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar that were considered to be presumptive *V. parahaemolyticus* were enumerated; $2.2x10^2$ CFU/ml was found in one shrimp pond water sample (P3). The results of environmental parameters and biological indicators were within the standard ranges prescribed by the guidelines of good practice for advanced shrimp culture, Department of Fisheries, Thailand. No bacteriophage specific to the shrimp pathogen *V. parahaemolyticus* was isolated from tested water samples using the double agar layer method. This preliminary study may benefit further alternative applications of bacteriophages to reduce the number of specific bacterial host cells in marine environments.

Keywords: *Vibrio parahaemolyticus,* Shrimp pond water, Estuarine water, Seawater, Environmental parameters, Bacteriophages

Introduction

Vibrio parahaemolyticus is a salt-requiring Gram-negative bacterium commonly found in seawater and seafood. It is a causative agent of acute gastroenteritis worldwide due to the consumption of raw or undercooked seafood. *V. parahaemolyticus* is divided into pathogenic and non-pathogenic strains. The pathogenic strain of *V. parahaemolyticus* produces hemolysin, namely, thermostable direct hemolysin (TDH) and/or TDH-related hemolysin (TRH), which are encoded by *tdh* and *trh,* respectively.[1]

In Thailand, *V. parahaemolyticus* is a leading cause of food-borne disease outbreaks followed by *Salmonella* spp., *Staphylococcus aureus, Clostridium botulinum,* and *C. perfringens,* respectively. Gastroenteritis cases infected by *V. parahaemolyticus* account for more than 50% of cases annually.[2] Generally, *V. parahaemolyticus* food poisoning in patients with mild symptoms is often self-limiting. Antimicrobial drugs, such as tetracycline, doxycycline, fluoroquinolones, gentamicin, and cefotaxime, are recommended for patients with severe symptoms.[3] However, ampicillin resistant and multi-drug resistant *V. parahaemolyticus* strains isolated from clinical and environmental samples have been reported.[4,5]

Recently, outbreaks of the newly emerged *V. parahaemolyticus* strain, which causes acute hepatopancreatic necrosis disease (AHPND) in cultivated shrimp, was first reported in China in 2009 and subsequently spread to many countries including Vietnam (2010), Malaysia (2011), Thailand (2012), and Mexico (2013). AHPND causes massive die-off of farmed shrimp. The affected shrimp exhibit atrophy and discoloration of the hepatopancreas.[6] The AHPND-causing strain has different virulence factors than the strain that causes acute gastroenteritis in humans. The *V. parahaemolyticus* strain that is pathogenic to shrimp carries toxin genes on its plasmid, namely, *pirA* and *pirB*.[7] Antimicrobial drugs have been applied to control the spread of aquaculture diseases, resulting in an increase in the number of antimicrobial- and multi-drug- resistant bacteria.[8]

In the aquaculture industry, antibiotics are commonly used to treat and prevent aquatic animal diseases.[9] Consequently, antimicrobial drug usage on shrimp farms may influence antimicrobial drug residues in the environment, such as in water and sediment, resulting in antimicrobial resistant bacteria.[10] In addition, *V. parahaemolyticus* is a normal flora in shrimp and in pond water used to rear shrimp.[11,12] The prevalence of antimicrobial resistant *Vibrio* spp. isolated from farmed marine shrimp has been published. *Vibrio* spp. is highly resistant to ampicillin (61%) and erythromycin (42%).[5] Recently, alternative applications of bacteriophages to reduce the number of specific host cells and antimicrobial resistant bacteria have been demonstrated. In particular, Vinod et al. isolated *V. harveyi* bacteriophage from shrimp pond water in order to develop it as a biocontrol for *V. harveyi* outbreaks in the aquaculture industry. The study by Vinod et al. revealed that shrimp affected by *V. harveyi* and subsequently treated with the bacteriophage showed higher survival rates compared to those treated with antibiotics.[13] Consequently, this study's objective was to determine the environmental parameters and isolate the bacteriophage specific to strains of the shrimp pathogen *V. parahaemolyticus* from shrimp pond water, estuarine water, and seawater samples collected in Surat Thani province, Thailand.

Methods

Sample Collection and Sampling Sites

This study analyzed ten marine water samples, namely, one estuarine water sample, three seawater samples, and six shrimp pond water samples. Seawater sampling sites were located in Bandon bay, a bay in the Gulf of Thailand in Surat Thani province (9°23'6"N99° 26'7"E, 9°23'8"N99°26'0"E and 9°27'9"N99°26'0"E). The sampling sites of estuarine water (9°14'13"N99°14'30"E) and shrimp pond water (9°12'20"N99°14'24"E, 9°13'20"N99°14'26"E, 9°12'48"N99°14'48"E, 9°12'57"N99°13'47"E, 9°13'10"N99°13'40"E, and 9°12'51"N99°13'36"E) were located in Punpin district, Surat Thani province, Thailand (Table 1, Figure 1). Shrimp pond water samples were collected during the no water-exchange cycle of shrimp rearing around 97-105 days. All water samples were collected during January 2019 by the grab sampling method using a Kemmerer depth sampler. One liter of each water sample was taken from 5 points at a depth of 1-meter. Samples were stored in cold and dark containers and transported to the Public Health Microbiology Laboratory at Suratthani Rajabhat University, Surat Thani, Thailand.

Measurement of Environmental Parameters

The environmental parameters of temperature, pH, dissolved oxygen (DO), oxidation-reduction potential (ORP), and salinity were used to determine the quality of marine water samples. These environmental parameters

Table 1 Environmental parameters of studied marine water samples

Source	Sample ID	Geographic coordinate	Environmental Parameters				
			Temperature (°C)	pH	DO (mg/L)	ORP (mV)	Salinity (ppt)
Suitable water quality for marine shrimp farm*			28–32	7.5–8.0	>5.0	-	2–35
Shrimp pond water	P1	9°12'20"N99°14'24"E	30.6	8.34	0.05	180	12
	P2	9°13'20"N99°14'26"E	30.1	8.35	0.00	180	5
	P3	9°12'48"N99°14'48"E	30.2	8.35	0.00	181	1
	P4	9°12'57"N99°13'47"E	30.4	8.63	4.21	180	3
	P5	9°13'10"N99°13'40"E	30.2	8.64	4.17	180	2
	P6	9°12'51"N99°13'36"E	30.4	8.64	4.45	180	3
Estuarine water	B1	9°14'13"N99°14'30"E	30.3	8.36	0.00	181	1
Seawater	M1	9°23'6"N99°26'7"E	25.6	7.26	1.15	36	30
	M2	9°23'8"N99°26'0"E	25.7	8.08	1.94	-27	29
	M3	9°27'9"N99°26'0"E	25.7	8.13	1.93	-30	29

* Suitable water quality for marine shrimp farm regarding guidelines of good practice for advanced shrimp culture by Department of Fisheries, Thailand; DO: dissolved oxygen; ORP: oxidation-reduction potential

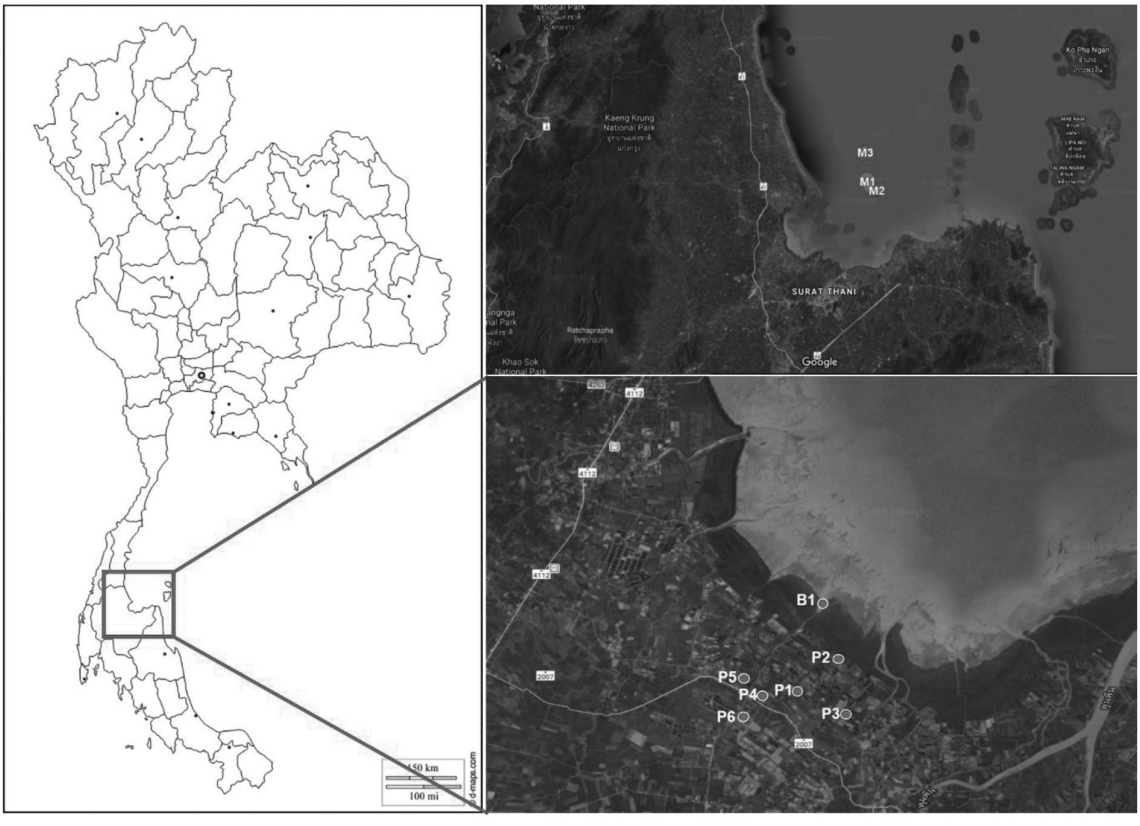

Figure 1 Sampling sites of shrimp pond water, estuarine water, and seawater samples

were measured on-site using the pH/mV/Conductivity/DO meter, model EC900 (Amtast, USA) during water collection.

Total Viable Count of Bacteria

Each water sample was tested for total viable count of bacteria using the spread plate technique. The total viable count method followed in-house laboratory methods as previously described.[14] Briefly, 0.1 ml of each water sample (10-fold dilution, starting 10^0-10^{-3}) was plated on plate count agar (PCA; Difco, BD, USA) with 1% NaCl and incubated at 37°C for 18-24 h. Triplicate plates for each dilution factor were performed. The number of bacteria grown on PCA with 1% NaCl between 25-250 colonies were counted. The standard plate count was calculated and noted as colony forming units per ml (CFU/ml).

Presumptive Vibrio Count

Each water sample was tested for presumptive *Vibrio* count using the spread plate technique. This method was adapted from the determination of *V. cholerae, V. parahaemolyticus,* and *V. vulnificus* from seafood using standard methods as previously described.[15] Briefly, 0.1 ml of each water sample (dilution factor, undiluted to 10^{-3}) was plated on Thiosulfate Citrate Bile Salt Sucrose (TCBS; Difco, BD, USA) medium and incubated at 37°C for 18-24 h. Triplicate plates for each dilution factor were performed. The numbers of green or yellow colonies grown on TCBS between 25-250 colonies were counted. Green colonies 2-4 mm in diameter were considered to be presumptive *V. parahaemolyticus.* The presumptive *Vibrio* count was calculated and noted as CFU/ml.

Isolation of Vibrio parahaemolyticus Bacteriophage

Media. All media used for bacteriophage isolation were prepared as described by Suthienkul.[16] Agar base (BA) and soft agar (SA) used for the isolation of the bacteriophage were prepared. The BA ingredients consisted of 1% polypeptone (Difco, BD, USA), 0.5% NaCl, and 1.3% Bacto agar (Difco, BD, USA); the final pH of 7.8 was adjusted. The SA ingredients were similar to BA, but 0.6% Bacto agar and 0.2% yeast extract were included. The BA plates were dried at 37°C before use. Peptone water (PW) containing 1% NaCl was used for bacterial cultures.

Bacteriophage isolation method. The ten water samples were examined for *V. parahaemolyticus* bacteriophage isolation using the double agar layer method as previously described by Suthienkul.[16] Briefly, 10 ml of each water sample was centrifuged at 17,300xg for 10 min at 4°C. The supernatant was filtrated using a filter paper with pore size of 0.45 µM (Millipore, USA). Each filtrated supernatant was tested with 10 strains of the shrimp pathogen *V. parahaemolyticus.* A 1 ml sample of each individual filtrated supernatant and 0.1 ml of each individual overnight culture of *V. parahaemolyticus* from 10 strains of the shrimp pathogen carrying *pirA* and *pirB* were mixed in 10 ml of PW with 1% NaCl and incubated at 37°C, overnight. Subsequently, 0.1 ml of chloroform was added to each overnight culture and then centrifuged at 17,300xg for 10 min at 4°C. The supernatant was collected and sterilized by membrane Millipore filtration and kept at 4°C until analysis.

The isolation of bacteriophage in filtrates using the double agar layer method was performed as follows: 0.1 ml of filtrate and 0.1 ml of bacterial culture were added on BA plates and covered with warmed SA containing 0.1% of 0.1M $CaCl_2$. The plates were totally mixed and incubated at 37°C overnight. A clear zone equated to the appearance of plaques and revealed as plaque forming units per ml (pfu/ml).

Results

Environmental Parameters of Water

The physical properties of water sample quality were determined using the environmental parameters of temperature, pH, DO, ORP, and salinity. As noted above, ten marine water samples consisting of six shrimp pond water samples, one estuarine water sample, and three seawater samples were tested. Table 1 shows the environmental parameters for each water sample. The temperature, pH, DO, ORP, and salinity of shrimp pond water samples ranged from 30.1°C-30.6°C, 8.34-8.64, 0.00-4.45 mg/L, 180-181 mV, and 1-12 ppt, respectively. The temperature, pH, DO, ORP, and salinity of estuarine water were 30.3°C, 8.36, 0.00 mg/L, 181 mV, and 1 ppt, respectively. The temperature, pH, DO, ORP, and salinity of seawater samples ranged from 25.6°C-25.7°C, 7.26-8.13, 1.15-1.93 mg/L, (-)30-36 mV, and 29-30 ppt, respectively. All measured environmental parameters for each setting were similar.

Total Viable and Presumptive Vibrio spp. Counts

Table 2 gives the number of total bacteria and

presumptive *Vibrio* spp. counts among the ten marine water samples. The total viable bacterial count of shrimp pond, estuarine, and seawater samples were 1.2-7.2 x10^3 CFU/ml, 1.2x10^4 CFU/ml, and 1.8-2.5 x10^6 CFU/ml, respectively.

Presumptive enumeration of *Vibrio* spp. on TCBS agar with green colonies of 2-4 in diameter was considered as presumptive *V. parahaemolyticus.* No green colonies were detected for nine marine water samples, namely, five shrimp pond water (P1, P2, P4, P5, P6), estuarine water, and three seawater samples. Presumptive *V. parahaemolyticus* count of those water samples were indicated as <10 CFU/ml. One shrimp pond water sample (P3) contained 2.2x10^2 CFU/ml of presumptive *V. parahaemolyticus* (Table 2).

Enumerations of total viable bacterial count and presumptive *Vibrio* spp. in seawater samples were higher than those in other tested water samples.

Table 2 Enumeration of total viable bacteria, presumptive *Vibrio* spp., and detection of *Vibrio parahaemolyticus* bacteriophage

Source	Sample ID	Total count (CFU/ml)	Presumptive *Vibrio* count (CFU/ml)		Presence of bacteriophage
			Yellow colony	Green colony	
Shrimp pond water	P1	7.2x10^3	< 10	< 10	Neg.
	P2	1.7x10^3	< 10	< 10	Neg.
	P3	1.3x10^3	2.1x10^2	2.2x10^2	Neg.
	P4	1.4x10^3	< 10	< 10	Neg.
	P5	1.2x10^3	< 10	< 10	Neg.
	P6	1.5x10^3	< 10	< 10	Neg.
Estuarine water	B1	1.2x10^4	< 10	< 10	Neg.
Seawater	M1	2.5x10^6	3.6x10^5	< 10	Neg.
	M2	2.0x10^6	3.2x10^5	< 10	Neg.
	M3	1.8x10^6	2.4x10^5	< 10	Neg.

CFU/ml = colony forming unit/ml; Neg. = negative

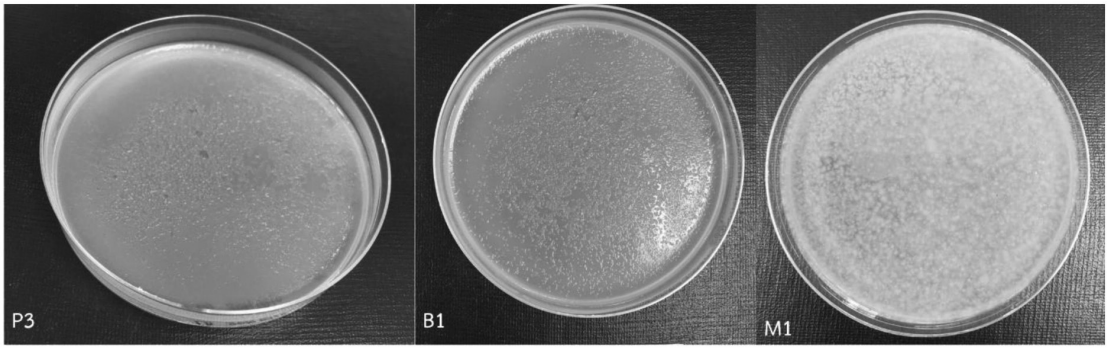

Figure 2 Detection of *Vibrio parahaemolyticus* bacteriophage

Isolation of Vibrio parahaemolyticus Bacteriophage

All ten marine water samples were negative for bacteriophage specific to strains of the shrimp pathogen *V. parahaemolyticus* (Table 2). Plaque was not observed on *V. parahaemolyticus* plates, which indicates no bacteriophage specific to the tested shrimp pathogen *V. parahaemolyticus* in this study (Figure 2).

Discussion

The measurement of water quality using the aforementioned environmental parameters indicated that the quality of shrimp pond water samples reached the guidelines of good practice for advanced shrimp culture as prescribed by the Department of Fisheries, Thailand. Specifically, pH should range between 7.5–8.2, concentration of DO should be high, salinity should be in a wide range between 2–35 ppt (0.2%–3.5% salinity), and ponds should be located away from sources of pollution.[17] Rearing pond water quality in this study was acceptable for shrimp hatcheries, though no dissolved oxygen was observed in the shrimp pond water samples of P1, P2 and P3. DO is free oxygen in water which is linked in biochemical and physiological activities.[18] In aquaculture systems, the concentration of DO should be above 3.0 mg/L to sustain shrimp cultures.[19] The lack of DO might be due to the high density of shrimp in grow-out ponds. In addition, algae growth in shrimp ponds uses oxygen at night time, and that might have affected the concentration of oxygen in the water samples that were collected during the day.

For the biological parameter, although *V. parahaemolyticus* is a normal flora of shrimp, the strain possessing *pirAB* on plasmid-affected cultivated shrimp with AHPND was reported.[7] In this study, one shrimp pond water sample (i.e., P3) contained presumptive *V. parahaemolyticus* at $2.2x10^2$ CFU/ml. The species specific and virulence genes of *V. parahaemolyticus* were not investigated in this study. However, pathogenic *V. parahaemolyticus* is rare; only 1–2% of pathogenic *V. parahaemolyticus* in humans have been found in environmental samples.[1] Similarly, the virulence genes of *V. parahaemolyticus,* including *tlh, tdh,* and *pirAB* that cause disease, were also not detected in shrimp pond water samples from Rembang Regency, Central Java, Indonesia.[20]

V. parahaemolyticus is a halophilic bacterium. The optimum temperature, pH, and salinity for *V. parahaemolyticus* are 30°C, 7.2, and 2.0%–4.0% NaCl, respectively.[21] In this study, low salinity in the range of 1–12 ppt or 0.1%–1.2% NaCl was observed in shrimp pond and estuarine water samples, which might not be an optimal concentration of salinity for the growth of *V. parahaemolyticus* and other halophilic *Vibrio* spp.. The reason why the salinity in shrimp pond and estuarine water samples was low is not known, but it might be related to the following. First, seasonal variation in salinity at Bandon bay may be due to fresh water from canals being discharged into the estuary, including rainfall during the sampling periods[22,23] and this water was also used for shrimp pond water. Second, the *V. parahaemolyticus* cell had entered the viable but non-cultural state when the salinity was lower than that required for optimal growth (3% NaCl).[24] Finally, the application of probiotics into shrimp cultural systems in order to increase the productivity of Pacific white shrimp may have suppressed *Vibrio* spp. growth.[25,26] Conversely, the enumeration of presumptive *V. parahaemolyticus* in seawater samples was higher than those of shrimp pond and estuarine water samples due to optimal environmental parameters.

V. parahaemolyticus bacteriophage is a lytic or virulent phage. It multiplies within bacterial cells by using host components to produce phage progeny to infect other bacterial cells.[27] Consequently, *V. parahaemolyticus* bacteriophage specifically infected only the *V. parahaemolyticus* host cell. In this study, no *V. parahaemolyticus* bacteriophage was isolated from water samples. Of the ten marine water samples, presumptive *V. parahaemolyticus* at $2.2x10^2$ CFU/ml was detected in one shrimp pond water sample. The negative isolation of bacteriophage from water samples might be due to the absence of specific host cells in water samples. A previous study reported the presence of *V. parahaemolyticus* cells was positive in oysters but negative in seawater samples.[28] Furthermore, *Vibrio* bacteriophages have been infrequently isolated from surface water. The high incidence bacteriophages found in marine mollusks, such as Pacific oysters, has indicated the replication of bacteriophages may occur within the intestines of these animals. No bacteriophage specific to pathogenic *V. parahaemolyticus* to humans was isolated.[29] Similarly, Tipluy collected 57 water samples from piers along the Chao Phraya river in Thailand. The results showed that pathogenic *V. cholerae* O1 was found in four water samples; however, *V. cholerae* bacteriophage was not detected in the 57 water samples.[30] Moreover, another

biocontrol of *V. parahaemolyticus,* i.e., *Bdellovibrio* spp., might eliminate *V. parahaemolyticus* cells or other vibrios and Gram-negative bacteria in environmental water.[31] Finally, the bacteriophages might be present in some study samples and lysed the environmental *V. parahaemolyticus* host cells other than the AHPND strains. In this study, the *V. parahaemolyticus* host cells were pathogenic to shrimp. However, more marine water samples and *V. parahaemolyticus* host cells, including those pathogenic to humans and shrimp, as well as non-pathogenic strains, may be included for further bacteriophage detection as an alternative approach for the biocontrol of *V. parahaemolyticus* in the environment.

Conclusion

Environmental parameters of shrimp pond water samples were suitable for cultivated shrimp. No bacteriophage specific to shrimp and pathogenic *V. parahaemolyticus* was observed from the ten water samples. For further bacteriophage detection, the authors recommend including a greater number of water samples from various environments and *V. parahaemolyticus* host cells, such as those pathogenic to humans and shrimp, as well as non-pathogenic strains. The success of bacteriophage isolation in the environment may have additional advantages for the prevention and control of pathogenic *V. parahaemolyticus* strains that cause foodborne disease outbreaks in humans, economic loss in the shrimp industry, and an increase in multidrug resistant strains via horizontal gene transfer in marine water samples.

Author Contributions

Kaknokrat Chonsin designed and performed experiments, analyzed data and co-wrote the paper. Bussayamas Hemmanee and Ratchakorn Hongkul performed experiments. Kanjana Changkaew and Pirom Noisumdaeng co-wrote the paper. Orasa Suthienkul supervised the research and manuscript preparation.

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Conflicts of interest

None

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