https://doi.org/10.48048/tis.2023.5962

Characterization and Genetic Relationships of *Vibrio* spp. Isolated from Seafood in Retail Markets, Yala, Thailand

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Received: 19 September 2022, Revised: 5 November 2022, Accepted: 24 December 2022, Published: 15 August 2023

Abstract

Vibrio spp. including *Vibrio cholerae* and *V. parahaemolyticus* are the major causative agent of diarrhea involved in seafood consumption. Yala is a southern border province in Thailand where no seafood sources are provided. Thus, it is necessary to import several seafood products from neighboring provinces. The risk of *Vibrio* spp. replication is increased during seafood transport. Therefore, the objectives of this study were to isolate and characterize *V. cholerae* and *V. parahaemolyticus* from seafood in Yala and to investigate their genetic relationships with virulent and clinical strains. A total of 100 *Vibrio* isolates were obtained from four different sample types in retail markets. It was found that two-thirds of all isolates were yellow colonies on TCBS agar. Using PCR with specific primers, 34 % of *V. cholerae* and 8 % of *V. parahaemolyticus* were identified. To classify the virulence strains, *Vibrio* spp. were positive for *hly*A and *tdh*, 17 and 1 %, respectively. Moreover, 54 % of *Vibrio* isolates were able to produce biofilm and 28 % of those isolates showed a high level of biofilm production. All representative strains of *V. cholerae* presented ß-hemolysin on blood agar correlated to *hly*A while representative strains of *V. parahaemolyticus* presented γ-hemolysin and ß-hemolysin on blood agar. In addition, ERIC-PCR provided different DNA profiles of representative *V. cholerae* and *V. parahaemolyticus* which some strains showed a correlation with clinical strains and virulent strains. Therefore, these findings might indicate the risk of *Vibrio* infections for seafood consumers, especially in Yala province where seafood needs to be imported from other provinces.

Keywords: *V. cholerae*, *V. parahaemolyticus*, Virulence genes, Hemolysin, Biofilm production, Genetic relationships

Introduction

Vibrio spp. are bacteria that inhabit in marine and estuarine areas [1]. There are several strains including both pathogenic and non-pathogenic strains, especially pathogenic strains which are the causative agents of gastrointestinal tract diseases. To illustrate, *V. cholerae*, is a causative agent of cholera and *V. parahaemolyticus*, is a causative agent of human acute gastroenteritis [1,2]. The pathogenicity of *Vibrio* spp. associated with various virulence factors such as toxin production, hemolysis activity, and biofilm formation. These virulence factors promote bacteria in pathogenicity and survival ability in environments such as marine water and sea animal surface [1,3]. Toxin production, thermostable direct hemolysin (TDH) is also found in *V. parahaemolyticus*. It is responsible for cytotoxicity and enterotoxicity [1,4]. Moreover, thermostable direct hemolysin-related hemolysin (TRH) is detected in *V. parahaemolyticus*, and hemolysin (HlyA) is found in *V. cholerae.* Both TRH and HlyA are involved in the lysis of erythrocytes and enterotoxicity, leading to the inflammation of the intestine [1,5,6]. Biofilm is a complex community of microorganisms embedded in a self-produced matrix and adhering to living surfaces, so this capacity enhances the environment fitness of *Vibrio* spp. [3,7,8].

There were continually several reports about the detections of *Vibrio* infections related to the consumption of raw or undercooked seafood in Thailand [1,3,8,9]. During 2014 - 2019, the reports showed that *V. parahaemolyticus* was the main causative agent of food poisoning in several areas in Thailand. In addition, cholera caused by *V. cholerae* has been reported repeatedly over the past 10 years; however, the patient rate has likely been decreased due to the improved sanitation facilities [10-15]. Interestingly, there are

reports about patients' cases caused by *Vibrio* infection in Yala province, which are non-seafood sources supplied [12] and necessary to import seafood products from nearby provinces. However, seafood transport might lead to a high risk of the prevalence of *Vibrio*. Therefore, the aims of this study were to detect *Vibrio* spp. isolates from seafood samples distributed in local markets, Mueang Yala, Yala, Thailand and to investigate the relationships among *Vibrio* isolates from seafood with virulent and clinical *Vibrio* spp. strains.

Materials and methods

Isolation of *Vibrio* spp. from seafood

A total of 33 seafood samples including shrimps, shellfish, squids, and crabs were collected from retail markets in Mueang Yala, Yala province. Twenty-five grams of seafood samples were crushed and mixed with alkaline peptone water (APW) before incubation at 42 °C for 6 h. Then a full loop of the sample was streaked on Thiosulfate citrate bile salt sucrose agar (TCBS) and incubated at 37 °C for 18 h. The yellow and green colonies were selected for the strain identification [16,17].

Identification of *V. cholerae* and *V. parahaemolyticus* by PCR

Bacterial DNA was extracted by a boiling method at 95 °C for 10 min [18]. Two sets of primers were used: *Omp*W for the detection of *V. cholerae* and *Tox*R for the detection of *V. parahaemolyticus* (**Table 1**). Then the PCR products were visualized after electrophoresis in a 1 % agarose gel [19,20].

Characterization of virulence factors in *V. cholerae* and *V. parahaemolyticus*

Detection of toxin genes

DNA of *V. cholerae* and *V. parahaemolyticus* was extracted by the boiling method at 95 °C for 10 min [18]. The detection of *hly*A and *tdh* genes was performed by the PCR method as previously described [21,22] using primers listed in **Table 1**.

Table 1 A list of primers used in this study.

Target genes	Primers	Nucleotide sequence (5'-3')	PCR product (bp)	References
*omp*W	OmpW-F OmpW-R	CACCAAGAAGGTGACTTTATTGTG GAACTTATAACCACCCGCG	588	[20]
*tox*R	tox R-F toxR-R	GTCTTCTGACGCAATCGTTG ATACGAGTGGTTGCTGTCATG	368	[21]
*hly*A	*hlyA*-F/clas *hlyA*-F/El Tor *hlyA*-R	GAG CCGGCA TTCATCTGAAT GGCAAACAG CGAAACAAATACC CTCAGCGGGCTA ATACGGTTTA	738/727 (clas) 481 (el)	[22]
tdh	tdh 1 tdh 2	GGTACTAAATGGCTGACATC CCACTACCACTCTCATATGC	251	[21]

Evaluation of biofilm formation

The bacteria were cultured in tryptic soy broth (TSB) + 1 % NaCl and incubated at 37 °C for 18 h. After the bacteria were adjusted to 0.5 McFarland standards, 100 µL of bacteria were added to a 96-well plate and incubated at 35 °C for 18 h. TSB + 1 % NaCl was used as negative control. Then, bacteria cells were measured using a microplate reader at OD_{600}. Deionized water was used to removed and washed each well for 3 times. The plate was stained with 0.1 % crystal violates for 30 min and washed with deionized water for 3 times. Then crystal violate was eluted using 95 % ethanol for 30 min. The concentration of biofilm was detected at OD_{570}. The volume of the biofilm was calculated by OD_{600}/ OD_{570}, and biofilm levels were classified according to the formula in **Table 2** [23,24]. The correlation analyses of biofilm ability and *Vibrio* strains were analyzed using the Chi-Square test. A p-value < 0.05 was considered to be statistically significant.

Table 2 Classification of biofilm levels.

Formulas	Levels of biofilm formation
$OD \leq OD_{cut}^{*}$	No
$OD_{cut}^{*} < OD \leq 2 \times OD_{cut}^{*}$	Low
$2 \times OD_{cut}^{*} < OD \leq 4 \times OD_{cut}^{*}$	Medium
$OD > 4 \times OD_{cut}^{*}$	High

*$OD_{cut} = OD_{avg}$ of Negative + 3×SD of Negative

Hemolysis assay

The representative bacteria were cultured in tryptic soy broth (TSB) + 1 % NaCl and incubated at 37 °C for 18 h. After that the bacteria were adjusted to 0.5 McFarland standards, the 10 µL of bacteria were dropped on blood agar and incubated at 37 °C for 18 h. The hemolytic activity was observed and classified as beta-hemolysin (completely hemolysis), alpha-hemolysin (partially hemolysis), and gamma-hemolysin (non-lysis) [25].

Evaluation of genetic relationships among *Vibrio* spp.

DNA extraction

DNA materials from representative strains and reference strains were extracted by DNA extraction Kits (Geneaid, Taiwan) and stored at –20 °C.

Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR)

ERIC-PCR was conducted using a pair of primers: ERIC 1 R (5 ' - ATGTAAGCTCCTGGG GATTCAC-3 ') and ERIC 2 (5 '-AAGTAAGTGACTGGGG TGAGCG-3 '). The 25 µL of ERIC-PCR reaction was composed of 2.5 µL 10X PCR buffer, 1.0 µL of each primer, 0.5 µL of 5 units of Takara Ex *Taq* DNA polymerase, and 2 µL of template DNA. ERIC-PCR condition was performed as follows; pre-denaturation at 95 °C for 7 min, followed by 30 cycles of denaturation at 90 °C for 30 s, annealing at 58 °C for 1 min, extension at 68 °C for 8 min, and a final extension at 68 °C for 16 min. PCR products were detected by electrophoresis in 1.5 % agarose gel at a voltage of 100 V cm^{-1} for 5 min, followed by 50 V cm^{-1} for 6 h. [26]. The dendrogram was constructed using a Bionumeric (Applied Maths, Sint-Martens-Latem, Belgium).

Results and discussion

A total of 100 *Vibrio* isolates were collected from 33 seafood samples: 20 shrimps, 7 shellfish, 3 squids, and 3 crabs sold in retail markets in Mueang Yala, Yala province. These isolates included 67 yellow and 33 green colonies (**Table 3**). The identification of *Vibrio* isolates via specific primers found 34 % *V. cholerae* and 8 % *V. parahaemolyticus*. The remaining strains were classified as non-*V. cholerae* and *V. parahaemolyticus* (Non VC-VP) (58 %) (**Figure 1**). This result indicated that most *Vibrio* spp. isolates were Non-*V. cholerae* and *V. parahaemolyticus*. Two pathogenic *Vibrio* spp.: *V. cholerae* and *V. parahaemolyticus* were found in this study. Over 40 % of isolates from seafood distributed in Mueang Yala, Yala province revealed the risk of *Vibrio* infections from non-seafood producers. The seafood transport from nearby provinces, Pattani and Narathiwat, play an important role in seafood distribution in Yala. Regarding, the seafood import to Yala, there is a high risk of bacteria replication in transfer periods which increases the hazards to consumers. There also were some reports of the presence of *Vibrio* spp. in seafood sold in Bangkok where the seafood had to import from other provinces [28,31]. Moreover, the results of this study were corresponded with several previous studies in several countries in Asia, which reported the detections of human pathogenic *Vibrio* spp. in shrimps, shellfish, and squids distributed in Malaysia, Vietnam, and Indonesia [27-31]. Previous studies reported that the prevalence of pathogenic *Vibrio* spp. was associated with seasons. Both *V. cholerae* and *V. parahaemolyticus* are more often detected during the rainy season. It may associate with the rainfall caused by the drainage of community waste contaminated with pathogenic *Vibrio* into seafood sources [32,33]. Consequently, the consumption of *Vibrio* spp. contaminated seafood is one way of *Vibrio* infections in humans. There have been continual reports of enteritis disease associated with the consumption of seafood, especially raw and undercooked seafood [1,8,9,12-15]. Moreover, the cross-contamination of *Vibrio* spp. in seafood can occur in the environment and the periods of carrying or selling to consumers. Moreover, previous studies have reported the contamination of *V. cholerae* in food related to cross-contamination by seafood storage and handling [34-36]. This study revealed that there was the presence of *V. cholerae* and *V. parahaemolyticus* in seafood

distributed in Mueang Yala, Yala province. Both *Vibrio* strains may be cross-contaminated from the environment and during seafood transport and handling. Thus, it revealed the risk of *Vibrio* infection in the non-seafood area.

Table 3 Colony characteristics of *Vibrio* spp. on TCBS agar.

Sample types (no.)	Colony characteristics on TCBS agar		Total
	Yellow	Green	
Shrimps (20)	40	22	62
Shellfish (7)	16	6	22
Squids (3)	9	0	9
Crabs (3)	2	5	7
Total	**67**	**33**	**100**

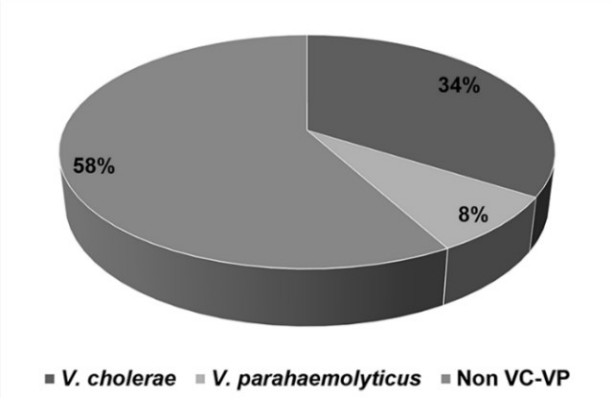

Figure 1 The prevalence of *V. cholerae*, *V. parahaemolyticus*, and non VC-VP in 100 *Vibrio* spp. isolates from seafood distributed in Mueang Yala, Yala province.

Both *hly*A and *tdh* encoded toxins were responsible for hemolysis activity. Characterization of toxin-producing genes in *Vibrio* spp. showed that *hly*A was found in 17 % of *Vibrio* spp., and *tdh* was detected in 1 % of *Vibrio* spp. In this study, 50 % of *V. cholerae* isolated from seafood contained *hly*A (**Figure 2**). The *V. parahaemolyticus* carrying *tdh* was pathogenic. This result was corresponded to a previous study which reported that *tdh* was a major virulence gene of pathogenic *V. parahaemolyticus* [1]. This result indicated that some *V. cholerae* and *V. parahaemolyticus* isolated from seafood distributed in Mueang Yala, Yala province contained the virulence genes, especially *V. cholerae*. Most of this detected strain presented *hly*A. Previous reports in Thailand and India where more than 80 % of *V. cholerae* isolated from environments such as seafood contained *hly*A [37,38].

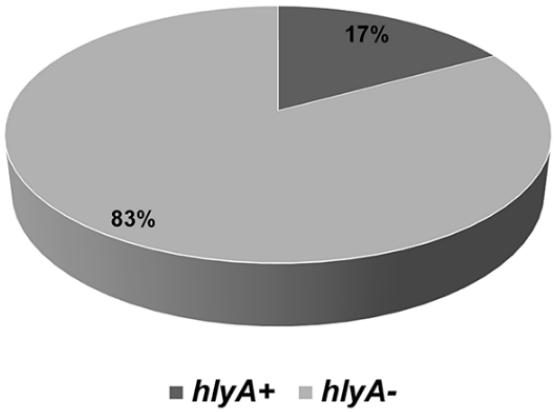

Figure 2 The distribution of *hly*A in *Vibrio* spp. isolates from seafood.

The result of the biofilm assay displayed that 54 % of *Vibrio* spp. isolates were able to produce the biofilm. The biofilm formation ability was classified into four groups including high, medium, low, and non-biofilm formation. 46, 28, 14 and 12 % of isolates were classified as non-biofilm, high, low, and medium biofilm formation, respectively (**Figure 3**). The analysis of the Chi-Square test showed that the variation of biofilm formation ability was independent of *Vibrio* spp. strains. (p-value = 0.118). Moreover, 50 % of isolates possessed high biofilm formation found in *V. cholerae* (**Figure 4**). Biofilm was considered a major factor that played a key role in the environmental fitness of bacteria [7,8]. Therefore, the various biofilm production ability of *Vibrio* spp. isolated from seafood might be a key factor in a virulence strain that promoted the risk of bacteria cross-contamination in animals and humans.

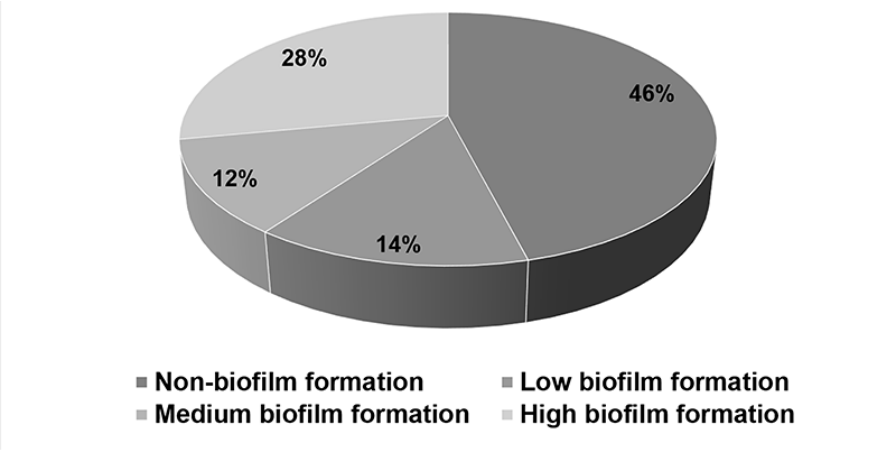

Figure 3 Percentage of *Vibrio* spp. in different groups of biofilm formation.

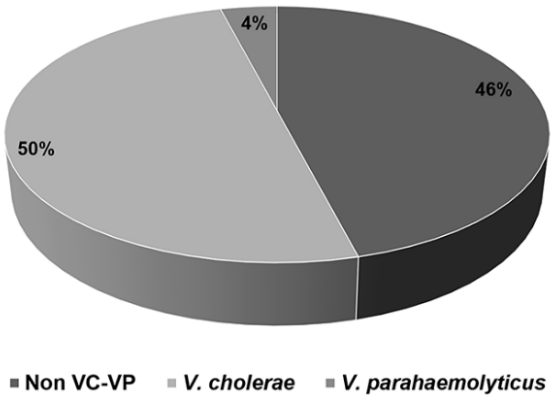

Figure 4 Percentage of *Vibrio* strains in high biofilm formation group.

Hemolysis activity on blood agar was evaluated in representative strains including 6 isolates of *V. cholerae* (*hly*A$^+$) and 4 isolates of *V. parahaemolyticus* (*tdh*$^+$/*tdh*$^-$) and reference strains including 6 isolates of *V. cholerae* and 4 isolates of *V. parahaemolyticus* (**Tables 4** and **5**). The results revealed that all *V. cholerae* isolates from seafood showed ß-hemolysin corresponding to *hly*A positive. The hemolysis activity was involved in toxin genes such as *hly*A and *stn*/*sto* [39]. The hemolysis activity with *hly*A gene usually found in *V. cholerae* O1, but rarely detected in *V. cholerae* non O1-non O139. Regarding hemolysis activity, both *V. cholerae* O1 and *V. cholerae* non O1-non O139 were classified as virulence strains [37,38]. In representative *V. parahaemolyticus*, 3 isolates including *tdh*$^+$/*tdh*$^-$ *V. parahaemolyticus* showed partial hemolysin as γ-hemolysin on blood agar. Interestingly, one isolate of *tdh*$^-$ *V. parahaemolyticus* showed complete hemolysin as ß-hemolysin (**Table 5**). This hemolysis activity might be involved in other toxin genes such as thermostable direct hemolysin-related hemolysin (TRH) and thermolabile hemolysin (TLH) [1,40]. The isolated *Vibrio* from seafood in Yala showed the representative strains of *V. cholerae* and *V. parahaemolyticus*; both of which strains carry major virulence factors of human pathogenic *Vibrio* spp. which can lyse erythrocytes. Therefore, the results indicated that these *Vibrio* isolated from seafood in Yala were virulent strains.

Table 4 Characteristics of representative *V. cholerae* isolated from seafood distributed in Mueang Yala, Yala and reference *V. cholerae*.

Isolates	Strains	Sources	Toxin genes	Biofilm formation*	Hemolytic activity on Blood agar
VCS1	*V. cholerae*	Squid	*hly*A$^+$	+++	ß-hemolysin
VCS2	*V. cholerae*	Shell	*hly*A$^+$	++	ß-hemolysin
VCS3	*V. cholerae*	Squid	*hly*A$^+$	+++	ß-hemolysin
VCS4	*V. cholerae*	Squid	*hly*A$^+$	+++	ß-hemolysin
VCS5	*V. cholerae*	Shrimp	*hly*A$^+$	0	ß-hemolysin
VCS6	*V. cholerae*	Shrimp	*hly*A$^+$	+++	ß-hemolysin
VCH7	*V. cholerae,* VCPSU1	Shrimp	*hly*A$^+$	ND	ß-hemolysin
VCH8	*V. cholerae,* VCPSU2	Shrimp	*hly*A$^+$	ND	ß-hemolysin
VCD1	*V. cholerae* O1, DMST16261	Clinical	*hly*A$^+$	ND	ß-hemolysin
VCD2	*V. cholerae* O1, DMST9700	Clinical	*hly*A$^+$	ND	α-hemolysin
VCD3	*V. cholerae* O139, DMST9701	Clinical	*hly*A$^+$	ND	α-hemolysin
VCD4	*V. cholerae* non O1-non O139, DMST2873	Clinical	*hly*A$^-$	ND	ß-hemolysin

*Biofilm formation: 0 = non biofilm formation, + = low biofilm formation, ++ = medium biofilm formation and +++ = high biofilm formation; ND = non detection

Table 5 Characteristics of representative *V. parahaemolyticus* isolated from seafood distributed in Mueang Yala, Yala and reference *V. parahaemolyticus*.

Isolates	Strains	Sources	Toxin genes	Biofilm formation*	Hemolytic activity on Blood agar
VPS1	*V. parahaemolyticus*	Shrimp	*tdh*-	+++	ß-hemolysin
VPS2	*V. parahaemolyticus*	Shrimp	*tdh*-	0	γ-hemolysin
VPS3	*V. parahaemolyticus*	Shrimp	*tdh*+	++	γ-hemolysin
VPS4	*V. parahaemolyticus*	Shrimp	*tdh*-	++	γ-hemolysin
VPC1	*V. parahaemolyticus* O3:K6, VPPSU1	Clinical	*tdh*$^+$	ND	α-hemolysin
VPC2	*V. parahaemolyticus* O1:KUT, VPPSU2	Clinical	*tdh*$^+$	ND	γ-hemolysin
VPC3	*V. parahaemolyticus* O1:K25, VPPSU3	Clinical	*tdh*$^+$	ND	γ-hemolysin
VPA1	*V. parahaemolyticus* ATCC17802	Clinical	*tdh*$^-$	ND	α-hemolysin

*Biofilm formation: 0 = non biofilm formation, + = low biofilm formation, ++ = medium biofilm formation and +++ = high biofilm formation; ND = non detection

The evaluation of the genetic relationships of *V. cholerae* from seafood (VCS1-6), virulent *V. cholerae* (VCH7-8), and *V. cholerae* DMST (*V. cholerae* O1 DMST16261, *V. cholerae* O1 DMST9700, *V. cholerae* O139 DMST9701, and *V. cholerae* non O1-non O139 DMST2873) using ERIC-PCR revealed that 6 isolates of *V. cholerae* from seafood products in Yala illustrated the difference DNA profiles according to 7 - 15 bands (**Figure 5**). Among the representative strains, the results showed high genetic variation, which was corresponded to previous studies of *V. cholerae* isolates from environmental samples including seafood samples in Indonesia, India, and Thailand [39,41,42]. Moreover, with 75 % similarity of DNA profiles, these strains were classified into 2 groups: C1 and C2. C1 contained 4 isolates, namely, (1)

V. cholerae VCS3, (2) *V. cholerae* O1 DMST16261, (3) *V. cholerae* O1 DMST9700, and (4) *V. cholerae* O139 DMST9701. *V. cholerae* VCS3 was isolated from seafood in Yala and presented the virulence factors: *hly*A^{+}, high biofilm production, and ß-hemolysin. *V. cholerae* O1 DMST16261, *V. cholerae* O1 DMST9700, and *V. cholerae* O139 DMST9701 were classified as the virulent strains and the causative agents of the pandemic [41]. C2 contained 2 isolates: (1) *V. cholerae* VCS1 and (2) *V. cholerae* non O1-non O139 DMST2873. *V. cholerae* VCS1 was isolated from seafood in Yala, and *V. cholerae* non O1-non O139 DMST2873 was usually found in the environment [39,41] . An ERIC-PCR result revealed that the genetic relationships of *V. cholerae*, isolated from seafood in Yala, were detected in both virulent strains and environmental strains, especially the isolates in the C1 group showed the cross-genetic relationships of the seafood isolates and the virulent strains (>75 % similarity). In addition, the result indicated that these strains were the virulent strains and might infect humans through seafood consumption. According to the evaluation of the genetic relationships of the representative *V. parahaemolyticus* from seafood (VPS1-VPS4), the pandemic *V. parahaemolyticus* (*tdh*$^{+}$, *trh*$^{-}$, GS^{+}: O3:K6, O1:KUT, and O1:K25), and *V. parahaemolyticus* ATCC17802 (VPA1), an ERIC-PCR result showed the different DNA patterns of all *V. parahaemolyticus* isolates including 5-14 DNA bands (**Figure 6**). At 75 % similarity of DNA profiles, *V. parahaemolyticus* isolates were classified into only 1 group: P1 which contained *V. parahaemolyticus* VPS3 and VPS4 isolated from seafood in Yala and *V. parahaemolyticus* VPC2 and VPC3 isolated from the patient. It could be possible that the representative *V. parahaemolyticus* had high genetic variation and some strains have a cross-genetic relationship with clinical strains (>80 % similarity). These results were corresponded to a previous study in Egypt that reported the cross-genetic relation among clinical and environmental *V. parahaemolyticus* isolates [43]. However, no correlation between DNA fingerprinting and hemolysis activity of *V. parahaemolyticus* isolates from seafood was found. The ERIC-PCR results of 10 representative strains of *Vibrio* spp. indicated the genetic variation among the isolates from seafood distributed in markets of Yala and the genetic relations of these isolates with some strains of virulent *Vibrio* spp. Besides, they might be infectious strains which transmitted to humans by seafood consumption.

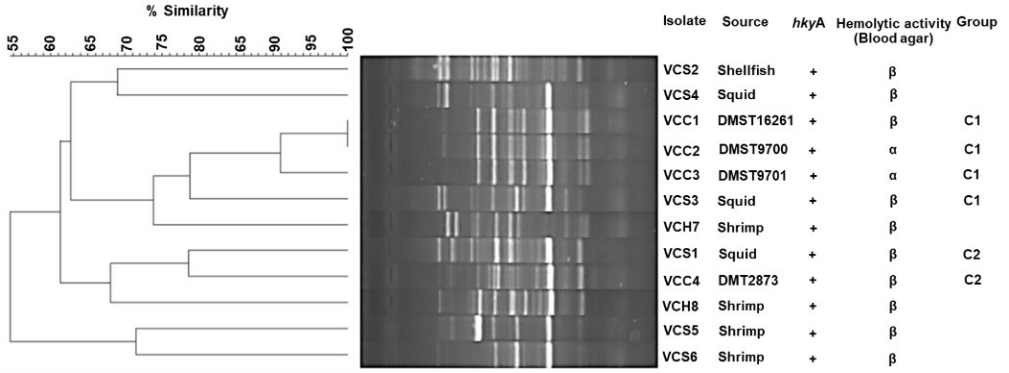

Figure 5 DNA fingerprints of 12 isolates of *V. cholerae* including representative *V. cholerae* isolates from seafood distributed in retail markets, Yala, Thailand, virulent strains and standard *V. cholerae* strains.

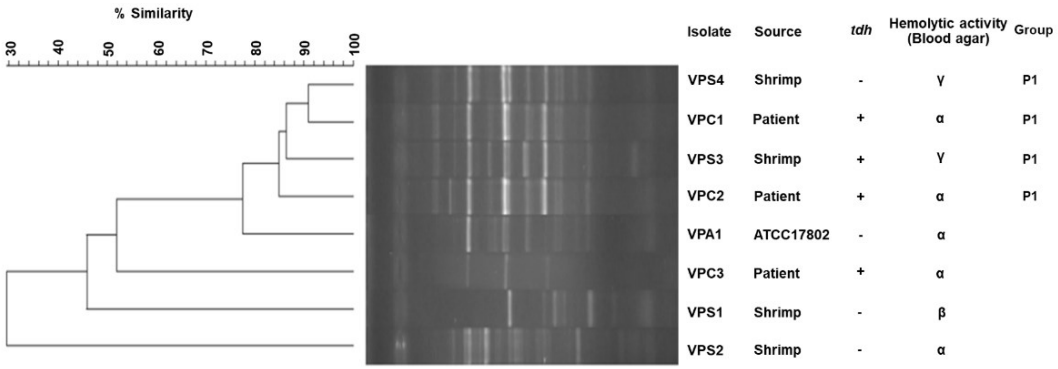

Figure 6 DNA fingerprints of 8 isolates of *V. parahaemolyticus* including representative *V. parahaemolyticus* isolates from seafood distributed in retail market, Yala, clinical strains and standard *V. parahaemolyticus* strains.

Conclusions

More than half of 100 *Vibrio* spp. isolated from seafood distribution in Mueng Yala, Yala appeared as yellow colonies on TCBS agar. Of 100 *Vibrio* spp. isolates, 2 potential virulent species were identified: *V. cholerae* (34 %) and *V. parahaemolyticus* (8 %). Moreover, 2 toxin-producing genes; *hly*A and *tdh* were detected in 17 and 1 % of *Vibrio* spp. isolates, respectively. The representative strains of *V. cholerae* and *V. parahaemolyticus* consisted of variant genetic relationships among different strains; some strains were related to clinical strains and presented virulence factors. In consequence, these findings indicated that the possibility of *Vibrio* spp. isolated from seafood contained virulent strains, which led to the increased risk of *Vibrio* infections in humans associated with the consumption of seafood.

Acknowledgments

This study was financially supported by Yala Rajabhat University (Contract no. บกศ.054/2564). We would like to thank Dr. Sompop Saeheng for his suggestion and proofreading English grammar. They also thank Ms. Krittika Dummunee and Publication Clinic, Faculty of Science Technology and Agriculture, Yala Rajabhat University for English language review.

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