ยีนก่อโรคของ *วิบริโอ พาราฮีโมไลติคัส* ที่แยกได้จากกุ้งและน้ำบ่อเลี้ยงกุ้งที่สัมพันธ์กับกลุ่มอาการตับและตับอ่อนตายเฉียบพลัน

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บทคัดย่อ

Vibrio parahaemolyticus เป็นแบคทีเรียที่อาศัยอยู่ในน้ำทะเล และเป็นสาเหตุการระบาดของโรคกระเพาะอาหารและลำไส้อักเสบเฉียบพลันในคนทั่วโลก ในปี ค.ศ. 2010 เกิดการอุบัติใหม่ของ *V. parahaemolyticus* สายพันธุ์ก่อโรคกลุ่มอาการตับและตับอ่อนตายเฉียบพลัน (AHPND) ในกุ้งเลี้ยง การระบาดของ AHPND ทำให้เกิดความเสียหายต่ออุตสาหกรรมการเลี้ยงกุ้งในหลายประเทศ *V. parahaemolyticus* สายพันธุ์ที่มียีน *pirA* และ *pirB* บนพลาสมิดจัดเป็นสายพันธุ์ก่อโรคดังกล่าวในกุ้ง การศึกษานี้ตรวจหา *V. parahaemolyticus* สายพันธุ์ที่ก่อโรคในคนที่มียีนสร้างสารพิษ *tdh* และ *trh* กับสายพันธุ์ก่อโรคในกุ้งที่มียีน *pirA* และ *pirB* จากกุ้งและน้ำบ่อเลี้ยงกุ้งที่สัมพันธ์กับ AHPND แบคทีเรียที่ใช้ในการศึกษาครั้งนี้คือ *V. parahaemolyticus* ที่เก็บรักษาในอาหารเลี้ยงเชื้อสำหรับเก็บรักษาจุลินทรีย์ จำนวน 83 ไอโซเลท ซึ่งแยกได้จากกุ้งเลี้ยง (n=28) และน้ำบ่อเลี้ยงกุ้ง (n=55) จากฟาร์มแห่งหนึ่งในภาคตะวันออกของประเทศไทย ซึ่งได้รับผลกระทบจากการระบาดของโรค AHPND ในปี ค.ศ. 2013 นำมาทดสอบจีโนไทป์ด้วยวิธี multiplex PCR ผลการศึกษาพบว่า *V. parahaemolyticus* ทั้งหมด 83 ไอโซเลท (ร้อยละ 100) ไม่มียีนก่อโรคในคน (tdh^-trh^-) และเมื่อทดสอบยีนก่อความรุนแรงของสายพันธุ์ก่อโรค AHPND พบว่า 74 ไอโซเลท (74/83, ร้อยละ 89.16) เป็นสายพันธุ์ $pirA^-pirB^-$ และ 9 ไอโซเลท (9/83, ร้อยละ 10.84) แยกเป็น 2 ไอโซเลท (2/28, ร้อยละ 7.14) จากกุ้ง 7 ไอโซเลท (7/55, ร้อยละ 12.73) จากน้ำบ่อเลี้ยงกุ้ง เป็นสายพันธุ์ $pirA^+pirB^+$ ผลการศึกษาพบ *V. parahaemolyticus* ที่มียีนก่อโรค AHPND จากทั้งตัวอย่างกุ้งและน้ำบ่อเลี้ยงกุ้ง ดังนั้นการตรวจติดตามยีนก่อความรุนแรงของโรคในสายพันธุ์อุบัติใหม่ของ *V. parahaemolyticus* จำเป็นสำหรับการเฝ้าระวังทางระบาดวิทยาและการตรวจติดตามทางสิ่งแวดล้อม

คำสำคัญ: *วิบริโอ พาราฮีโมไลติคัส* กลุ่มอาการตับและตับอ่อนตายเฉียบพลัน ยีน *tdh* ยีน *trh* ยีน *pirA* ยีน *pirB* ยีนก่อความรุนแรง

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Virulence genes of *Vibrio parahaemolyticus* isolated from acute hepatopancreatic necrosis disease associated with shrimp and grow-out pond water

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Abstract

Vibrio parahaemolyticus, a marine bacterium, is an etiologic agent of acute gastroenteritis disease outbreaks among people worldwide. A newly emerged *V. parahaemolyticus* caused acute hepatopancreatic necrosis disease (AHPND) among cultivated shrimp occurred in 2010. The AHPND outbreak caused major shrimp industry losses in many countries. The *V. parahaemolyticus* strain carried toxin genes (i.e., *pirA* and *pirB*) on its plasmid is pathogenic to shrimp. This study investigated the presence of human pathogenic genes, *tdh* and *trh*, and shrimp pathogenic genes, *pirA* and *pirB*, from AHPND related samples. A preserved stock culture of 83 *V. parahaemolyticus* were isolates from the AHPND affected white shrimp (n=28) and grow-out pond water (n=55) were examined. The tested strains were originally isolated from white shrimp and rearing pond water samples of one single private farm located in Eastern Thailand the AHPND outbreak in 2013. The individual isolates were tested using the genotyping method by multiplex PCR. The results revealed that all 83 (100%) *V. parahaemolyticus* isolates were lacking human pathogenic virulence genes (tdh^-trh^-). When examined using the virulence markers of the AHPND causing strain, 74 (74/83, 89.16%) isolates were $pirA^-pirB^-$ strains and nine (9/83, 10.84%) isolates (two [2/28, 7.14%] white shrimp and seven [7/55, 12.73%] grow-out pond water isolates) were $pirA^+pirB^+$ strains. This finding showed that *V. parahaemolyticus* with AHPND-associated genes presented in both shrimp and grow-out pond water. Consequently, probing the virulence genes of newly emerged *V. parahaemolyticus* strains would be essential for epidemiological surveillance and environmental monitoring.

Keywords: *Vibrio parahaemolyticus*, acute hepatopancreatic necrosis disease, *tdh*, *trh*, *pirA*, *pirB*, virulence gene

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Introduction

Vibrio parahaemolyticus is a seafood-borne pathogen that causes acute gastroenteritis in humans who consume raw or undercooked seafood. It is a Gram-negative, rod-shaped, facultative anaerobic and halophilic bacterium that is commonly found in estuarine and marine environments. The human pathogenic strain produces thermostable direct hemolysin (TDH) and/or TDH-related hemolysin (TRH) that are encoded by *tdh* and *trh*, respectively. The non-pathogenic strain to humans lacks both hemolysins.[1] The majority of the human pathogenic *V. parahaemolyticus* strain is recovered from clinical samples while that of the non-pathogenic strain is found in environmental samples including seafood and seawater.[2,3,4] *V. parahaemolyticus* infection is a public health concern for many countries including Thailand.[5]

During 2009-2013, *V. parahaemolyticus* caused acute hepatopancreatic necrosis disease (AHPND) outbreaks in farmed shrimp, thus rising socioeconomic concerns for many Asian countries including Thailand.[6] This shrimp pathogenic strain produces binary *Photorhabdus* insect-related (Pir) toxins, $PirAB^{vp}$ that are encoded by *pirA* and *pirB*.[7] Both *pirA* and *pirB* are located on the plasmid DNA of the *V. parahaemolyticus* AHPND-causing strain.[7,8,9] The $PirAB^{vp}$ toxins cause AHPND which leads to massive cell sloughing of hepatopancreas (HP) tubule epithelial cells together with the dysfunction of B, F, R and E-cells of HP in affected shrimp.[8,10]

This study investigated human and shrimp pathogenic virulence genes in *V. parahaemolyticus* isolates from cultivated Pacific white shrimp and their grow-out pond water associated with AHPND outbreak were examined to determine the virulence characteristics of the isolates.

Objectives

1. To investigate the human pathogenic virulence genes, namely *tdh* and *trh*, in *V. parahaemolyticus* isolated from acute hepatopancreatic necrosis disease associated shrimp and their grow-out pond water.

2. To determine the shrimp pathogenic virulence genes, namely *pirA* and *pirB*, in *V. parahaemolyticus* isolated from acute hepatopancreatic necrosis disease associated shrimp and their grow-out pond water.

Materials and Methods

Bacterial strains

A total of 83 *V. parahaemolyticus* isolates obtained from preserved stock cultures and kept in semi-solid stock medium containing 1% NaCl were used in this study. Theses tested isolates were retrieved from our stock collection belonging to Professor Orasa Suthienkul. Twenty-eight and 55 *V. parahaemolyticus* isolates were collected from Pacific white shrimp and their grow-out pond water, respectively, from a farm affected by AHPND in Eastern Thailand in 2013 (Table 1). All archived *V. parahaemolyticus* isolates were re-subcultured on selective thiosulfate citrate bile salt sucrose (TCBS; Eiken, Tokyo, Japan)

agar and colony morphology was observed for culture purity as previously described.[11,12] In case of contamination, cultures were biochemically characterized according to a method described elsewhere.[13] Working stock cultures were prepared using tryptic soy agar (TSA; Difco, Detroit, MI) slant containing 3% NaCl. Subsequently, virulence genes analysis was carried out. The *V. parahaemolyticus* strain VP156 (*ldh*$^+$*tdh*$^+$*trh*$^+$) was used as a positive control for human pathogenic virulence genes detection. The AHPND-causing strain VP-E61[14] (*pirA*$^+$*pirB*$^+$) was used as a positive control for determination of shrimp pathogenic virulence genes.

Table 1 *Vibrio parahaemolyticus* isolates used in this study

Grow-out pond	No. of *V. parahaemolyticus* isolates		Total (N = 83)
	Pacific white shrimp	Grow-out pond water	
Pond 1	10	19	29
Pond 2	8	17	25
Pond 3	10	19	29
	28	**55**	**83**

DNA preparation

DNA from *V. parahaemolyticus* was extracted using the rapid boiled-lysate method. Briefly, a 1,000 µl aliquot of overnight bacterial culture in LB broth containing 3% NaCl was centrifuged at 12,000*x* g for 5 min. The pellet was re-suspended in 400 µl of TE buffer and centrifuged at 12,000*x* g for 5 min. Two hundred microliter of 1% Chelex in TE buffer was added to the bacterial cell pellet. The tube was incubated at 65°C for 30 min and then boiled for 10 min. The tube was then placed on ice for 10 min and centrifuged at 16,000*x* g for 10 min. The supernatant was used as the DNA template for PCR assays.

Detection of human pathogenic virulence genes

The presence of human pathogenic virulence genes including *ldh, tdh* and *trh* was determined using three sets of primers (Table 2). The lecithin-dependent hemolysin gene (*ldh*), also named thermolabile hemolysin gene (*tlh*), is species-specific gene for *V. parahaemolyticus* and was used as internal control for PCR analysis in this study. One microliter of each sample DNA was used as the template for PCR detection. PCR condition was performed as previously described by Chonsin *et al*.[15] PCR products were analyzed using 2% agarose gel electrophoresis.

Detection of shrimp pathogenic virulence genes

The presence of shrimp pathogenic virulence genes including *pirA* and *pirB* was determined using two sets of primers (Table 2). One microliter of each sample DNA was used as the template for PCR detection. PCR condition was adapted from a previous study described by Han *et al*.[7] PCR products were analyzed using 2% agarose gel electrophoresis.

Table 2 Nucleotide primers used in this study

Genes	Primers (5'->3')	Amplicons size (bp)	Tm (°C)	Reference
ldh	F-AAA GCG GAT TAT GCA GAA GCA CTG R-GCT ACT TTC TAG CAT TTT CTC TGC	450	61 59	22
tdh	F-GTA CCG ATA TTT TGC AAA R-ATG TTG AAG CTG TAC TTG A	382	48 53	25
trh	F-CTC TAC TTT GCT TTC AGT R-TAC CGT TAT ATA GGC GCT TA	276	50 56	26
pirA	F-TGA CTA TTC TCA CGA TTG GAC TG R-CAC GAC TAG CGC CAT TGT TA	284	61 58	7
pirB	F-TGA TGA AGT GAT GGG TGC TC R-TGT AAG CGC CGT TTA ACT CA	392	58 56	7

bp: base pair, Tm: melting temperature

Gel electrophoresis

Presence of PCR products was examined by gel electrophoresis. The 5 µl of each PCR product was mixed with 5 µl of 3X GelRed™ Nucleic gel stain (Biotium, Hayward, CA, USA). Each PCR product was loaded into individual well of 2% agarose gel (Promega, Madison, USA). The PCR products were electrophoresed on agarose gel in horizontal electrophoresis tank (Advance, Japan) containing 1X Tris/Borate/EDTA (TBE) buffer (Takara, Japan) at 100 voltages for 30 min. The 0.26 µg (2 µl) of 100 bp DNA ladder (Nippon Gene-Wako, Japan) was used as a standard size marker. The DNA bands were visualized under UV light using Illuminator PrepOne™ Sapphire (Embi Tec, San Diego, CA, USA).

Statistical analysis

The descriptive statistics including frequency and percentage were used in this study to describe the presence of human and shrimp pathogenic virulence genes among *V. parahaemolyticus* isolates from AHPND-associated shrimp and grow-out pond water samples.

Results

Detection of human pathogenic virulence genes

The 83 isolates of *V. parahaemolyticus* from Pacific white shrimp (n = 28) and grow-out pond water (n = 55) were examined for the presence of human pathogenic virulence genes *tdh* and *trh* by multiplex PCR. When the presence of human pathogenic virulence genes was examined, the result revealed that none of the tested isolates carried *tdh* or *trh* genes (Table 3). Figure 1 shows the analysis of PCR product by 2% of agarose gel electrophoresis. The human pathogenic strain isolated from acute gastroenteritis patient, VP156 ($ldh^+tdh^+trh^+$), was used as positive control. All *V. parahaemolyticus* isolates recovered from AHPND-associated shrimp and grow-out pond water samples were positive for species-specific gene (*ldh*) but all were negative for virulence genes, *tdh* and *trh*.

Table 3 The presence of human pathogenic virulence genes among *Vibrio parahaemolyticus* tested isolates

Genes	No. (%) of isolates positive for hemolysin genes		Total (N = 83)
	Pacific white shrimp (n = 28)	Grow-out pond water (n = 55)	
ldh	28 (100)	55 (100)	83 (100)
tdh	0 (0)	0 (0)	0 (0)
trh	0 (0)	0 (0)	0 (0)

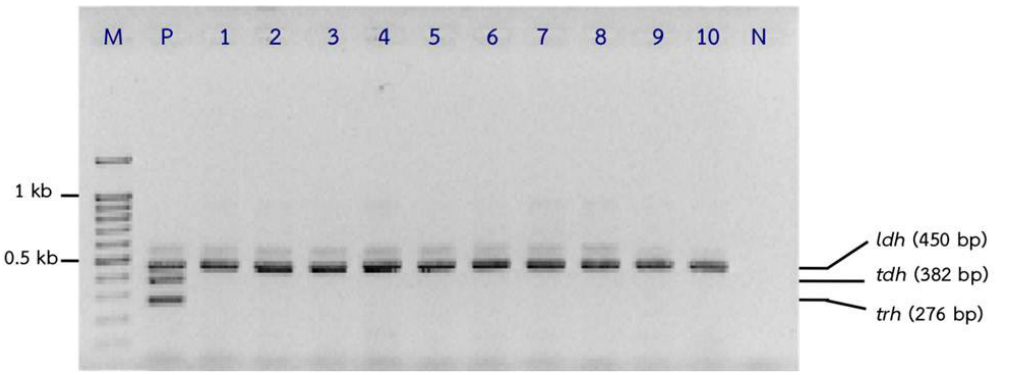

Figure 1 The 2% agarose gel electrophoresis of amplification specificity of heat labile hemolysin gene (*ldh*), thermostable direct hemolysin gene (*tdh*), and *tdh*-related hemolysin gene (*trh*) among *V. parahaemolyticus* isolates from Pacific white shrimp and grow-out pond water using multiplex PCR; In lane M: DNA molecular size marker (100-bp marker); lane 1-5: *V. parahaemolyticus* isolates from Pacific white shrimp; lane 6-10: *V. parahaemolyticus* isolates from grow-out pond water; lane P: positive control strain VP156 ($ldh^+tdh^+trh^+$); lane N: negative control (no DNA template)

Detection of shrimp pathogenic virulence genes

The 83 isolates of *V. parahaemolyticus* from Pacific white shrimp (n = 28) and grow-out pond water (n = 55) were examined for the presence of shrimp pathogenic virulence genes, *pirA* and *pirB*, by multiplex PCR. Of 83 isolates, nine (10.84%) of 83 isolates were positive for *pirA* and *pirB* while 74 (89.16%) isolates were lacking *pirA* and *pirB* (Table 4). Of 28 Pacific white shrimp isolates, two (7.14%) were positive for *pirA* and *pirB*. Of 55 grow-out pond water isolates, 7 (12.73%) were $pirA^{+}pirB^{+}$ strains (Table 4, Figure 2).

In order to proof that the presence of *pirA* and *pirB* was specific to AHPND-causing strains, 10 *V. parahaemolyticus* strains obtained from AHPND-associated (VP-E73, VP-E74, VP-E76, VP-E79, VP-E86), environmental (VP-E70, VP-E77, VP-E83), and clinical (VP-P1, VP-P30) samples as previously reported by Chonsin *et al*.[15] were analyzed. The result illustrated that the presence of *pirA* and *pirB* was specific to AHPND-causing strains (Figure 3).

Table 4 The presence of shrimp pathogenic genes among *Vibrio parahaemolyticus* tested isolates

Genes	No. (%) of isolates positive for genes		Total (N = 83)
	Pacific white shrimp (n = 28)	Grow-out pond water (n = 55)	
Distribution of each gene			
ldh	28 (100)	55 (100)	83 (100)
pirA	2 (7.14)	7 (12.73)	9 (10.84)
pirB	2 (7.14)	7 (12.73)	9 (10.84)
Distribution of genes in each combination			
$ldh^{+}pirA^{-}pirB^{-}$	26 (92.86)	48 (87.27)	74 (89.16)
$ldh^{+}pirA^{+}pirB^{+}$	2 (7.14)	7 (12.73)	9 (10.84)
	28 (100)	**55 (100)**	**83 (100)**

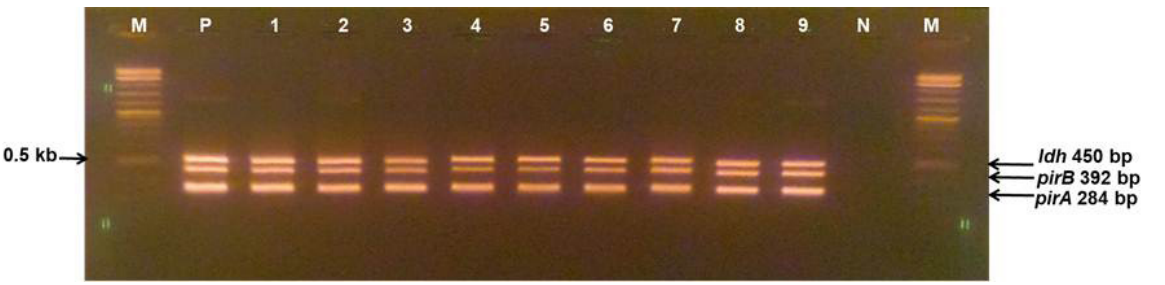

Figure 2 The 2% agarose gel electrophoresis of amplification of *pirA* and *pirB* using multiplex PCR; In lane M: DNA molecular size marker (2-log DNA ladder); (a) lane 1-2: *V. parahaemolyticus* isolates from Pacific white shrimp Vp225, Vp346; (b) lane 3-9: *V. parahaemolyticus* isolates from grow-out pond water Vp474, Vp476, Vp477, Vp478, Vp480, Vp481, Vp482; lane P: positive control (VP-E61 strain $pirA^{+}pirB^{+}$); lane N: negative control (no DNA template)

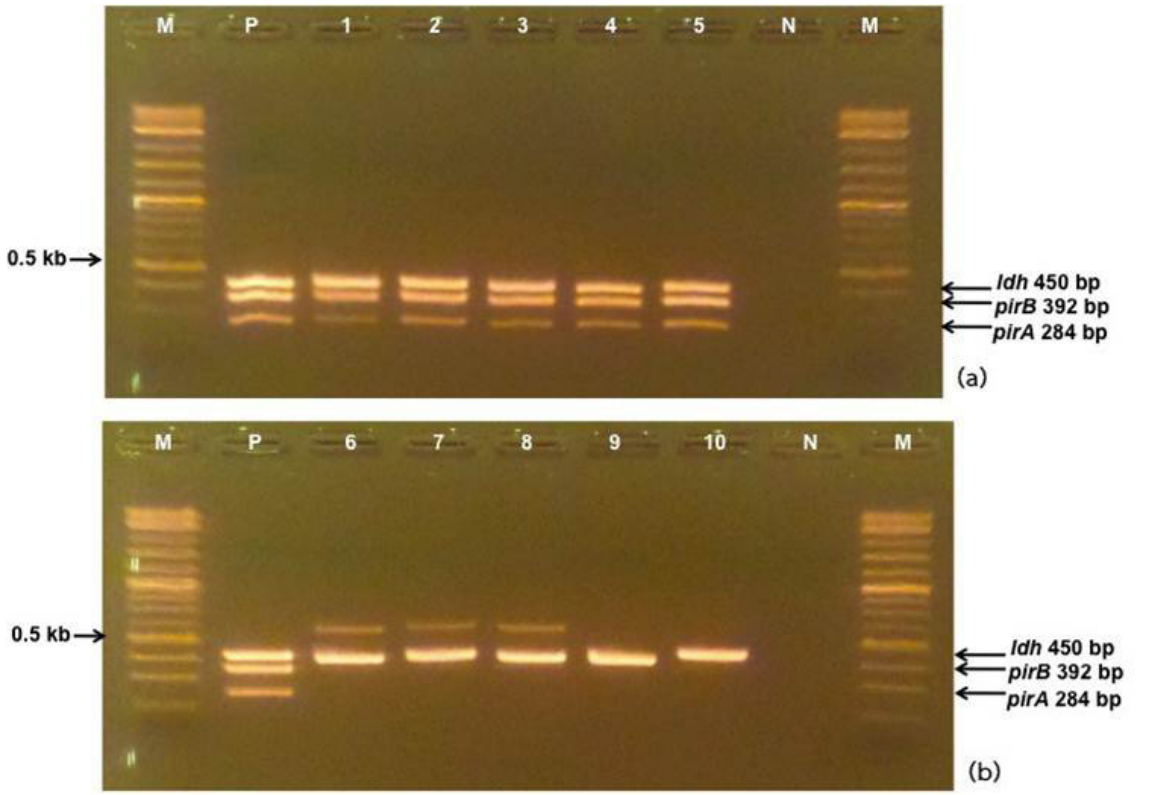

Figure 3 The 2% agarose gel electrophoresis of amplification of *pirA* and *pirB* using multiplex PCR; In lane M: DNA molecular size marker (2-log DNA ladder); (a) lane 1-5: AHPND causing strains; (b) lane 6-10: *V. parahaemolyticus* isolates from clinical and environmental samples; lane P: positive control (VP-E61 strain $pirA^{+}pirB^{+}$); lane N: negative control (no DNA template)

Discussion

The human pathogenic *V. parahaemolyticus* strain (tdh^{+} and/or trh^{+}) is a causative agent for acute gastroenteritis in humans. Since the toxigenic strains of *V. parahaemolyticus* have been detected in environmental samples[16], investigating the *tdh* and *trh* of *V. parahaemolyticus* isolates from AHPND samples is necessary. This study implies that none of the tested samples contained the human pathogenic genes ($tdh^{-}trh^{-}$ strain). Similarly, when Joshi *et al*. characterized the human pathogenic virulence genes among four AHPND-causing strains, none of these strains contained both *tdh* and *trh*.[10] In addition, another previous study in Southern Thailand reported that all 33 AHPND-associated isolates lacked both *tdh* and *trh* genes.[17] This finding might suggest that *V. parahaemolyticus* isolates recovered from AHPND affected shrimp and their grow-out pond water were environmental strains ($tdh^{-}trh^{-}$ genotype) that were not associated with disease in humans.

Generally, 90% of seafood samples were positive for *V. parahaemolyticus*[16] and most of them were environmental strains (*tdh*$^-$*trh*$^-$ genotype).[5] The AHPND-causing strain is an opportunistic marine shrimp pathogen that acquired plasmid containing *pirA* and *pirB* genes from environment.[8] In this study, 10.84% (9/83) of *V. parahaemolyticus* isolates were *pirA*$^+$*pirB*$^+$ genotype. Similarly, the prevalence of *V. parahaemolyticus* causing AHPND in shrimp (9.9%), molluscan shellfish (0.7%) and shrimp pond water (4.8%) samples has been reported from Vietnam.[18] AHPND-causing strains carry the specific *pirA* and *pirB*. Originally, *pirA* and *pirB* are found in *Photorhabdus luminescents* bacteria and encoded for PirAB toxin which indicates the insecticidal activity to *Spodophtera exigua* larvae.[19] The *pirA* and *pirB* genes are located on the self-transmissible plasmid of AHPND-causing *V. parahaemolyticus* stains.[7,8,9] Lee *et al.* suggested that the loss of *pirAB* gene from the plasmid could change the shrimp pathogenic strain into a non-pathogenic strain.[8] In this study, *pirAB*-positive *V. parahaemolyticus* strains were recovered from grow-out pond water. It might be possible that there were some environmental factors in the grow-out pond that induced horizontal gene transfer between *V. parahaemolyticus* and other bacteria. Previous studies have shown that environmental factors, such as salinity and nutrients, induced the horizontal gene transfer of bacterial cells.[20,21,22] Recently, the outbreaks of AHPND in farmed shrimp have been reported from Chinese Taipei and Japan in 2019 and 2020, respectively.[23,24] In conclusion, the monitoring of *pirA* and *pirB* genes in grow-out pond water might initially prevent the outbreak of AHPND in shrimp farm.

Conclusions

This study found the *pirAB*-positive strains of *V. parahaemolyticus* recovered from cultivated Pacific white shrimp and grow-out pond water. Consequently, probing the virulence factors of newly emerged *V. parahaemolyticus* strains would be essential for epidemiological surveillance and environmental monitoring.

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