Diagnosis, genetic variations, virulence, and toxicity of AHPND-positive *Vibrio parahaemolyticus* in *Penaeus monodon*

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Abstract

Acute hepatopancreatic necrosis disease (AHPND) is an emerging shrimp (*Penaeus monodon*) disease caused by *Vibrio parahaemolyticus* (VP) since 2013 in Bangladesh. The aim of this work was to evaluate a PCR and RT-PCR techniques as rapid methods for detecting *V. parahaemolyticus* AHPND-positive *P. monodon* using genetic markers. Healthy and diseased shrimp (*P. monodon*) samples were collected from three monitoring stations. The samples were enriched in TCBS plates and DNA extraction from the cultured bacteria. DNA quantifications, PCR amplification, RT-PCR, and gene sequencing were done for the detection of *V. parahaemolyticus* AHPND-positive *P. monodon*. The sequence of PCR amplicons showed 100% identity and significant alignment with *V. parahaemolyticus*. The primers used provided high specificity for *V. parahaemolyticus* in PCR detection compared with another *Vibrio* species. In the PCR, amplification resulted positive amplicons, whereas, non-AHPND isolates showed no amplicons. Neighbor-joining methods indicated that all genes evolved from a common ancestor and clades have different traits with very low genetic distance and low variability. The pairwise alignment scores of *atpA*, *tox*, *blaCARB*, 16S rRNA, and *pirA* genes were 100.0, 98.90, 98.89, 95.53, and 41.42, respectively. The RT-qPCR exposed variable expression levels for all genes in the AHPND-positive strain. Homology analysis and distance matrix exhibited all genes to have the lowest similarity and most divergence, offering the highest specificity. In this study, the expression and variability of target genes confirmed the presence of *V. parahaemolyticus* in all sampling sites. The results suggested that PCR amplification, RT-qPCR, and gene sequencing can be used for the rapid detection of *V. parahaemolyticus* in AHPND-positive *P. monodon* that may lead to subsequent prevention and treatment research in the future for managing this disease.

Keywords Diagnosis · *Penaeus monodon* · *Vibrio parahaemolyticus* · Acute hepatopancreatic necrosis disease (AHPND genetic variations, virulence, and toxic gene)

Introduction

Vibriosis is one of the major disease problems in shellfish and finfish aquaculture responsible for the mortality of cultured shrimp worldwide (Mohney et al. 1994). Opportunistic pathogens

with highly virulent strains (Ishimaru et al. 2009) include *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio harveyi* and *Vibrio parahaemolyticus* (Kannapiran et al. 2009). In an initial outbreak in the southwest of China in 2009, acute hepatopancreatic necrosis disease (AHPND) had its rapid, lethal effect in early stages of shrimp and had spread all over the world. Over the past 40 years, Asia's farmed shrimp aquaculture industry is impacted by episodes of disease, notably AHPND, resulting in substantial national income losses (Shinn et al. 2018a). *V. parahaemolyticus* is widely distributed in marine environments and frequently isolated from a variety of raw seafood, particularly shellfish. The *V. parahaemolyticus* harboring virulence genes such as *tdh* and *trh* (Zhang and Orth 2013) have toxic effect on human health.

AHPND is characterized by high mortality generally when shrimp is 1 month old or post larvae is around 20–30 days old (De Schryver et al. 2014); it is responsible for a significant proportion of economic losses recorded from at least eight Asian territories. An empty gut and an atrophied, pale hepatopancreas (Lai et al. 2015), massive sloughing off of the epithelial cells of the hepatopancreas (Sirikharin et al. 2015), and hemocyte infiltration are caused by a specific set of *V. parahaemolyticus* (De Schryver et al. 2014).

AHPND was first detected in shrimp farms in southwestern Bangladesh in 2013 and consequently spread to other cultural areas of Bangladesh. The pathogen is transferred orally and then localizes at the shrimp gastrointestinal tract and creates a poison that causes tissue devastation and invalidism of the shrimp digestive system known as the hepatopancreas (Zorriehzahra and Banaederakhshan 2013). To control AHPND, farmers implemented prevention through pond eradication and renovation, but it could not stop outbreaks of the pathogen once the disease appeared repeatedly in shrimp farms. To prevent AHPND, its causative agent (Praveena et al. 2014) namely *V. parahaemolyticus* must be characterized thoroughly. Although plasmid-encoded binary toxins *pirA* and *pirB* (Kim et al. 2015) are found to be the primary causes of AHPND, whether other virulent factors are *toxR* and *toxA* genes (Kim et al. 1999; Sirikharin et al. 2015) commonly present in *V. parahaemolyticus* and may play essential roles in genetic diversity analyzed by 16S rRNA gene (Feng et al. 2017) during shrimp infection remains unknown (Li et al. 2017).

The PCR primers target specific DNA sequences using PCR present in samples in several locations, this tool identified pathogenic *V. parahaemolyticus* causing AHPND (Han et al. 2015a). The primer set AP1 and AP2 which target the DNA sequences of AHPND was the first PCR diagnostic tool used in 2013 (Flegel and Lo 2014a). Three sets of AHPND primers (AP1, AP2, and AP3), AP2 showed a false positive result due to the plasmid mutation lacking the toxin gene (Flegel and Lo 2014b). Among (AP1, AP2, and AP3) the primer sets, AP3 demonstrated the highest sensitivity and best specificity (Flegel 2014). The TUMSAT-Vp3 primer used targeting the AHPND DNA sequences (Tinwongger et al. 2014). The AP4 nested PCR method showed higher sensitivity and amplified *pirA* and *pirB* genes in *V. parahaemolyticus* and detected AHPND isolates containing these two genes (Dangtip et al. 2015). Among the recent PCR methods, AP3 method is considered the most promising tool for detecting AHPND which targets the *pirA* as it exhibited high sensitivity and specificity (Soto-Rodriguez et al. 2015). Thus, the use of AP3 primers which can detect *pirA* gene and duplex PCR method to detect *pirA* and *pirB* genes is the most recommended confirmatory tests for AHPND detection (Han et al. 2015b). The current disease problems in Asia, notably AHPND caused by pathogenic isolates of *Vibrio parahaemolyticus* (Shinn et al. 2018b). In 2020, Santos et al. (2020) reported the different strategies in diagnosing and potential treatments for AHPND and the discovery of pathogenic mechanisms involved in AHPND.

This study aimed to analyze the specific detection, genetic variability, and expression of virulence and toxic genes using rapid detection method in *V. parahaemolyticus* isolated from *Penaeus monodon* in affected areas in Bangladesh remain unclear, which may include the essential groundwork for advance research of AHPND.

Materials and methods

Isolation and storage of *Vibrio parahaemolyticus* isolates

AHPND-causative *Vibrio parahaemolyticus* was isolated from shrimp culture ponds or ghers in PCF Feed Industries Ltd. in Bagherhat, C.P. Bangladesh Co., Ltd. in Khulna, and Radiant Shrimp Farms Ltd. in Sathkhira respectively. Dead and moribund juvenile (~2 g) shrimps (*Penaeus monodon*) were collected from 30 AHPND outbreaks ponds or ghers. About 25 g of each type of sample (gills, guts, and hepatopancreas) was thoroughly triturated in a sterile mortar and pestle with use of 225 ml of alkaline saline peptone water (ISO) (Thermo Scientific™) and homogenized for 2 min in a stomacher (Life Technologies, CA, USA) (Chonsin et al. 2015). Tenfold serial dilution of 1 ml of homogenates was established in alkaline saline peptone water (ASPW⁺) with three replicates which were generated and shaken at 170 rpm at 37 °C for 18 h. A total of 100 µl volumes of the extract was plated onto thiosulfate-citrate-bile salts-sucrose (TCBS) plates (Remel Inc., Santa Fe Drive, Lenexa, USA) for 24 h. The *Vibrio parahaemolyticus* sucrose agar (VPSA) incubated at 37 °C for 24 h. The characteristic large colonies (3–4 mm) with light blue or green centers on TCBS and VPSA were regarded as presumptive *V. parahaemolyticus* and further subjected to morphological, cultural, and biochemical characterization. A series of biochemical tests as per BAM, USDA method (Kaysner and DePaola 2004) was used for the identification of *Vibrio* isolates.

All colonies were picked and streaked on tryptone soya agar (TSA) (Sigma-Aldrich, Germany) supplemented with 3% (w/v) NaCl (Sigma-Aldrich, Germany). After 24 h incubation, one colony from each plate was inoculated into tryptone soya broth (TSB) (Sigma-Aldrich, Germany) enriched with 3% (w/v) NaCl grown by shaking at 170 rpm at 37 °C for 12 h. The bacterial suspension was incubated at 37 °C for 12 h with continuous shaking. The total 12 h culture volume (50 ml) was used as inoculum for 500 ml fresh medium (500 ml) followed by continued cultivation with shaking until the OD₆₀₀ reached 0.6 (approximately 6–8 h) and was equivalent to approximately 2×10^8 cells per ml. The bacterial cells were removed by centrifugation at 8500 rpm for 10 min at 4 °C; the supernatant was used to prepare crude protein fractions. Subsequently, 1 ml of medium was mixed with 1 ml of 50% (v/v) ultrapure glycerol (Invitrogen™) and stored at –80 °C. A total of 1 ml bacterial cells of overnight culture was centrifuged, and the pellet was suspended in 400 µl of ddH₂O. The bacterial suspension was centrifuged at 11,000 rpm for 6 min. The supernatant was used as DNA template for PCR assay.

Bacterial genomic and plasmid DNA extraction

A general DNA extraction technique was applied to obtain the DNA of 30 isolates from healthy/suspected/infected shrimp stomach or hepatopancreatic tissue. Bacterial genomic DNA and plasmid DNA were applied with DNAzol™ reagent to isolate genomic DNA (a vast spectrum, fast isolation, and large recovery of genomic DNA kits, Invitrogen™, and PureLinkR Pro Quick96 Plasmid Kit, Invitrogen™), following the manufacturer's instruction.

In this study, 0.01–1 ng of DNA extracted from bacterial isolate or 10–100 ng from shrimp tissue produces the volume of template DNA in the 25 µl PCR reaction.

Primers used for PCR amplification

The well-conserved regulatory genes *toxR* and *toxA* (Sirikharin et al. 2015), a highly conserved 16S rRNA gene (Tarr et al. 2007), a novel species-specific genetic marker *blaCARB-17* (Li et al. 2016), AP1 and AP2 (Feng et al. 2017) and *pirA* gene (AP3) (Sirikharin et al. 2014), was analyzed and examined for PCR amplification to produce the expression of virulence and toxic genes *tlh*, *tdh*, and *trh* of AHPND *V. parahaemolyticus* isolates (Gutierrez West et al. 2013). The sequence of primers applied for the amplification of target genes is listed in Table 1.

Table 1 The sequence of primer pairs (forward and reverse) for PCR amplification of *trh*, *tdh*, 16S rRNA, *toxR*, *atpA*, *tlh*, *pirA*, *toxA*, *pirB*, and *blaCARB-17* targeting genes

Primer pair	Oligonucleotide sequence (5'-3')	Product length	Target gene	Reference
Trh-F	CTCTACTTTGCTTTTCAGT	460 bp	<i>trh</i>	(Suthienkul et al. 1995)
Trh-R	AATATTCTGGAGTTTCAT			
Tdh-F	CGTTGATTATTCTTTTACGA	623 bp	<i>tdh</i>	(Karunasagar et al. 1996)
Tdh-R	TTTGTGGATATACACAT			
V.16S-700F	CGGTGAAATGCGTAGAGA T	663 bp	16S rRNA	(Tarr et al. 2007)
V.16S-1325R	TTACTAGCGATTCCGAGTTC			
ToxB-F	TGTACTGTTGAACGCCTA	503 bp	<i>toxR</i>	(Neogi et al. 2010)
ToxB-R	CACGTTCTCATACGAGTG			
atpA-VP-F	TACTAGGCCGCGTAGTA	794 bp	<i>atpA</i>	(Izumiya et al. 2011)
atpA-VP-R	CGCTGGACGTACACCT			
AP1-F	CCTTGGGTGTGCTTAGAGGATG	700 bp		(Flegel and Lo 2013)
AP1-R	GCAAACATATCGCGCAGAACACC			
AP2-F	TCACCCGAATGCTCGCTTGTGG	700 bp		(Flegel and Lo 2013)
AP2-R	CGTCGCTACTGTCTAGCTGAAG			
TLH-F	AAAGCGGATTATGCAGAAGCACTG	450 bp	<i>tlh</i>	(Dickinson et al. 2013)
TLH-R	GCTACTTTCTAGCATTTTCTCTGC			
AP3-F	ATGAGTAACAATATAAAACA	333 bp	<i>pirA</i>	(Sirikharin et al. 2014)
AP3-R	TGAAAC			
Tt16AP4-F1*	GTGGTAATAGATTGTACAGAA	1269 bp	<i>toxA</i>	(Dangtip et al. 2015)
AP4-R1	ATGAGTAACAATATAAAACA			
	TGAAAC	230 bp	<i>toxA</i>	(Dangtip et al. 2015)
AP4-F2	ACGATTTTCGACGTTCCCCAA			
AP4-R2	TTGAGAATACGGGACGTGGG	284 bp	<i>pirA</i>	(Han et al. 2015b)
PIR-F1	GTTAGTCATGTGAGCACCTTC			
AP4-R1	TGACTATTACGATTGGACTG	392 bp	<i>pirB</i>	(Han et al. 2015b)
PIR-F2	CACGACTAGCCATTGTTA			
PIR-R2	TGATGAAGTGATGGGTGCTC	135 bp	<i>pirA</i>	(Han et al. 2015b)
qPCR-F	TGTAAGCGCCGTTAACTCA			
qPCR-R	TTGGACTGTGGAACCAACG			
	GCACCCCATTTGGTATTGAATG			
	Probe:			
	AGACAGCAAACATACACCTA			
	TCATCCCGCA			
CARB-F	ACC(T)TTGATGGAAGATA	303 bp	<i>blaCARB-17</i>	(Li et al. 2016)
CARB-R	T(C)TAACTTTCTTGTAGTGC(A)			

*Note that primer AP4-F1 is identical to primer AP3-F from the AP3 method

PCR amplification of target genes

The template DNA derived from AHPND-suspected or positive 30 isolates was used for PCR amplification (Sirikharin et al. 2014) with specific primer set for particular target genes in Table 1. The final reaction volume was 10 µl, containing 5 µl of Green Master Mix (Go-TagG2), 1 µl of 10 µM primers, 1 µl of template DNA, and 3 µl nuclease-free water. The thermal profile for DNA amplification in a thermal cycler (Professional standard gradient, Biometra, Germany) was as follows: preheating at 95 °C for 2 min and 1 min denaturation at 94 °C for 35 cycles. The annealing temperature was 58 °C (primer-specific) for 1 min and then was elongated or extended to 72 °C for 1 min to a final extension of 72 °C for 5 min. Moreover, 1% agarose gel was used to separate the amplified PCR product from each sample electrophoretically (Nacalai Tesque, Inc., Kyoto, Japan) at a constant voltage of 120 for 30 min in the 1 × TAE buffer. The gel was stained with 10 µl ethidium bromide (10 mg/mL in 100 mL of DW) for 10 min and visualized under ultraviolet (UV) light, and molecular weight marker was 1 Kb plus DNA ladder (Invitrogen™, Thermo Fisher Scientific). The DNA bands were observed on high-performance UV transilluminator and photographed with a gel documentation system (BioDoc-It™ Imaging system, Cambridge, UK). The sequences (next-generation sequencing platform, 1st Base Laboratories, Malaysia) of the PCR amplicons were analyzed by MEGA X (version 10.0.5) (Kumar et al. 2018), DNAMAN (Lynnon Biosoft Corporation, USA, version 10.0.2.100) (Austin 2010) and Geneious prime software (Biomatters Limited, New Zealand, version 2020.0.2). The NCBI-BLAST (GeneBank) and SMART BLAST were used for sequence analysis (Table 2) and the specificity is shown in Table 3. The multiple sequence alignments were monitored using Geneious prime, ClustalW, and DNAMAN software. The neighbor-joining method (Saitou and Nei 1987) and the maximum composite likelihood method (Tamura et al. 2004) were used to determine the evolutionary history and distance analyzed by MEGA X.

Quantitative PCR for cDNA synthesis

The RNA was extracted using the Ambion RNAqueous Midi Kit (Invitrogen™) from TSB+ (Sigma-Aldrich, Germany) culture medium via centrifugation at 8000g for 10 min in accordance with the instruction. Thermo Scientific™ Culti-Loops™ *V. parahaemolyticus* ATCC™ 17802™ was used as a negative control. Thermo Scientific Maxima H Minus cDNA Synthesis Master Mix with dsDNase provides a simple workflow that combines genomic DNA elimination and cDNA synthesis in a one-tube procedure. The cDNA reaction components are pre-mixed into a complete master mix that is convenient to use, reduces pipeting steps, and is optimized for cDNA synthesis in two-step quantitative RT-PCR (RT-qPCR) applications.

Homology tree of target genes within AHPND-positive isolates

The DNAMAN software (Lynnon Biosoft Corporation, USA, version 10.0.2.100) was used to analyze the homology of target genes within AHPND-positive isolates by applying an effective alignment method. The NCBI nucleotide collection (nr) and whole-genome shotgun (wgs) database (accession nos.: AP014860.1, LK021128.1, CP021148.1, and CP033146.1) were used to retrieve target genes *toxR*, 16S rRNA, AP1, AP2, *pirA* (AP3), *toxA*, *blaCARB-17*, *tlh*, *atpA*, *tdh*, and *trh*-like sequences. The sequence of the target genes was used to construct the homology trees.

Table 2 The amplification of PCR result of AP1, AP2, *toxR*, 16S rRNA, *pirA* (AP3), *toxA*, *blaCARB*–17, *tlh*, *atpA*, *tdh*, and *trh* in AHPND-positive isolates

Bacterial isolates	Primer pair/target gene											Identity* (%)
	<i>toxR</i>	16S rRNA	AP1	AP2	AP3	<i>toxA</i>	<i>blaCARB</i> –17	<i>tlh</i>	<i>atpA</i>	<i>tdh</i>	<i>trh</i>	
ahpn-1	+	+	–	+	+	–	+	+	+	–	–	100
ahpn-3	+	+	+	+	+	+	+	+	+	+	+	100
ahpn-8	+	+	+	+	+	+	+	+	+	+	+	100
ahpn-11	+	+	+	+	+	+	+	+	+	–	–	100
ahpn-12	+	+	+	–	+	+	+	+	+	–	–	100
ahpn-13	+	+	+	+	+	+	+	+	–	+	+	99
ahpn-14	+	+	+	+	+	+	+	+	+	+	–	99
ahpn-15	+	+	+	+	+	+	+	+	+	+	+	100
ahpn-16	+	+	+	+	+	–	+	+	+	+	+	99
ahpn-17	+	+	+	+	+	+	+	+	+	+	+	99
ahpn-18	+	+	–	+	+	+	+	+	+	+	–	99
ahpn-19	+	+	+	+	+	–	+	+	+	–	–	99
ahpn-20	+	+	+	+	+	+	+	+	+	+	+	100
ahpn-21	+	+	+	+	+	–	+	+	+	–	–	99
ahpn-22	+	+	+	+	+	+	+	+	+	–	–	99
ahpn-23	+	+	+	+	+	+	+	+	–	+	+	99
ahpn-24	+	+	–	+	+	+	+	+	+	+	+	99
ahpn-25	+	+	+	+	+	+	+	+	+	+	+	100
ahpn-26	+	+	+	+	+	+	+	+	+	–	+	100
ahpn-27	+	+	+	–	+	–	+	+	–	–	–	99
ahpn-28	+	+	+	–	+	–	+	+	–	–	–	99
ahpn-29	+	+	+	+	+	+	+	+	–	+	+	100
ahpn-30	+	+	+	+	+	+	+	+	+	+	+	100

*Summary of metadata for genome sequence using BLAST (nr) in NCBI producing species identity of 30 isolates collected from major shrimp farming regions of Bangladesh

Expression analysis of target genes by qPCR

The primer pair (qPCR-forward and qPCR-reverse, and probe) in Table 1 was chosen to quantify *pirA* gene expression. ATCC17802 was used as the negative control and 16S rRNA gene were selected for the normalization of gene expression in RT-qPCR. Every 1 µl of cDNA was used as template. The final reaction volume of 20 µl consisted of 10 µl of Syber Green Master Mix (Thermo Fisher Scientific), 1.6 µl of 10 mM forward and reverse primer, 0.4 µl of ROX Reference Dye (Invitrogen™), 2 µl of cDNA template, and 6 µl of ddH₂O for RT-qPCR (7500 Fast Real-time PCR system, Applied Biosystems). The thermal profile was as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 15 s. Each reaction was carried out in triplicate and analyzed by ABI 7500 v2.0.6. software.

Results

Among the 30 AHPND outbreak shrimp ponds or ghers yielded a total of 30 isolates that were stored at –80 °C. The sequence of PCR amplicon analysis of the 30 isolates yielded 23 *V. parahaemolyticus* positive samples that produced 100% identity and significant alignment with *V. parahaemolyticus*. The other 7 isolates corresponded to common marine

microorganisms that produced 99% significant alignment with *Photobacterium* and *V. harveyi* (Table 2). In the initial screening, 23 isolates provided a positive signal with *toxR*, 16S rRNA, *pirA*, *blaCARB-17*, and *tlh*. The primers confirmed that six isolates isolated from shrimp in growing ponds (ahpn-3, ahpn-8, ahpn-15, ahpn-20, ahpn-25, and ahpn-30) contained *toxR*, 16S rRNA, AP1, AP2, *pirA* (AP3), *toxA*, *blaCARB-17*, *tlh*, *atpA*, *tdh*, and *trh* genes, but not in the other 17 isolates (Table 2). The PCR technique determined the specificity of target genes compared with separate bacterial isolates in Table 3; 100% specificity was provided in *V. parahaemolyticus* with *toxR*, *toxA*, 16S rRNA, *pirA*, *blaCARB-17*, and *tlh*. The *atpA*, *tdh*, and *trh* genes yielded 83%, 77%, and 85% specificities in *V. parahaemolyticus*, respectively, whereas the 30 strains were isolated from shrimp in growing ponds. *V. alginolyticus* isolated from shrimp yielded 66% and 33% specificities with 16S rRNA and *pirA* genes, respectively. The other *Vibrio* spp. isolated from food and ATCC sources, *Pseudomonas* and *Enterobacter* isolated from clinical sources and *Aeromonas*, *Escherichia coli*, *Salmonella*, and *Staphylococcus* isolated from food sources showed 0% specificity with *toxR*, *toxA*, 16S rRNA, *pirA* (AP3), *blaCARB-17*, *tlh*, *atpA*, *tdh*, and *trh* (Table 3).

Agarose gel (1%) was used to reveal the PCR amplification of target genes *toxR*, 16S rRNA, AP1, AP2, *pirA* (AP3), *toxA*, *blaCARB-17*, *tlh*, *atpA*, *tdh*, and *trh* by using AHPND-positive representative strain ahpn-25 (the samples of ahpn-3, ahpn-8, ahpn-15, ahpn-20, ahpn-25, and ahpn-30 showed 100% identity in Table 2 and revealed all target genes by PCR in Table 2; among them, ahpn-25 was used as a representative strain) to identify *V. parahaemolyticus* in Fig. 1. Lanes 3–13 show the bands of positive amplicons at 350, 663, 700, 700, 336, 333, 303, 450, 794, 623, and 460 bp, whereas non-AHPND (ahpn-2) exhibited no amplicons in lane 2.

Table 3 The PCR methods were resulting in the specificity targeting different genes in AHPND-positive and AHPND-negative isolates

Species	Source	No. of isolates	Positive rate (%)									
			<i>toxR</i>	<i>toxA</i>	16S rRNA	<i>pirA</i>	<i>blaCARB</i> −17	<i>tlh</i>	<i>atpA</i>	<i>tdh</i>	<i>trh</i>	
<i>Vibrio parahaemolyticus</i>	Shrimp	30	100	100	100	100	100	100	83	77	85	
<i>V. harveyi</i>	Shrimp	1	0	0	0	0	0	0	0	0	0	
<i>V. campbellii</i>	Shrimp	1	0	0	0	0	0	0	0	0	0	
<i>V. anguillarum</i>	Shrimp	3	0	0	66	33	0	0	0	0	0	
<i>V. vulnificus</i>	Food	1	0	0	0	0	0	0	0	0	0	
<i>V. cholerae</i>	Food	5	0	0	0	80	0	0	0	0	0	
<i>V. metschnikovii</i>	Food	1	0	0	0	0	0	0	0	0	0	
<i>V. fluvialis</i>	ATCC *	1	0	0	0	0	0	0	0	0	0	
<i>V. mimicus</i>	ATCC	1	0	0	0	0	0	0	0	0	0	
<i>V. natriegens</i>	Food	1	0	0	0	0	0	0	0	0	0	
<i>Pseudomonas</i> sp.	Clinical	1	0	0	0	0	0	0	0	0	0	
<i>Aeromonas</i> sp.	Food	1	0	0	0	0	0	0	0	0	0	
<i>Escherichia coli</i>	Food	2	0	0	0	0	0	0	0	0	0	
<i>Salmonella</i> sp.	Food	2	0	0	0	0	0	0	0	0	0	
<i>Staphylococcus</i> sp.	Food	2	0	0	0	0	0	0	0	0	0	
<i>Enterobacter</i> sp.	Clinical	1	0	0	0	0	0	0	0	0	0	

*The American Type Culture Collection

The amplified PCR products of the target genes with six AHPND-positive representative isolates (ahpn-3, 8, 15, 20, 25, and 30) received 99% identity (NCBI, BLAST) with their closed sequence in Table 2 and Fig. 2a and exhibited high homology. To construct the phylogenetic relationship (Li et al. 2016), 8 *Vibrio* spp. were selected from GeneBank to compare with target gene sequences; all genes were derived from a common ancestor (Fig. 2a). All genes showed a common ancestor (accession no. CP022245.1), whereas all clades were derived with different traits with very low genetic distance (Fig. 2b). The sequences of the PCR amplification of target genes (*toxR*, 16S rRNA, *pirA* (AP3), *toxA*, *blaCARB-17*, *tlh*, *atpA*, *tdh*, and *trh*) from an AHPND-positive representative strain (ahpn-25) were aligned with the reference strain of *V. parahaemolyticus* (accession no. CP034571.1) with low variability (Fig. 3).

The PCR amplification, agarose gel electrophoresis, and RT-qPCR assay of target genes 16S rRNA, *blaCARB-17* and *atpA* revealed accurate amplified fragment in Fig. 1 and comparatively high expression and constantly maintained 100% specificity in Table 2 for *V. parahaemolyticus* (Izumiya et al. 2011; Dickinson et al. 2013) in parallel with different isolates in Table 3. The unique homology was found for target genes (16S rRNA, *tox*, *blaCARB-17*, *pirA*, *atpA*, *tlh*, *tdh*, and *trh*) compared with another *Vibrio* spp. The degrees of similarity ranges were (23–58%) for *pirA*, *trh*, *blaCARB-17*, *tlh*, 16sRNA, *tlh*, *tdh*, and *tox* genes in *V. parahaemolyticus*, respectively, compared with the *atpA* gene (Fig. 4). The *trh* revealed the highest distance of 0.736 and the lowest distance involved with the *tdh* gene was 0.000, showing the low divergence and lowest specificity for detection of *tdh* (Fig. 5).

The expression levels of the *pirA* gene, the virulence gene of AHPND-positive isolates, after incubation for 4 h at 37 °C was determined. This result showed that the *pirA* gene was expressed in all AHPND-positive isolates, but that levels of expression were variable (Fig. 6).

Discussion

In this study, highly conserved detection gene 16S rRNA (Tarr et al. 2007), *atpA* (Izumiya et al. 2011), *blaCARB-17* (Li et al. 2016), virulence gene *pirA* (Sirikharin et al. 2014; Han

Fig. 1 The results of the PCR fingerprints, *trh*, *tdh*, 16S rRNA, *toxR*, *atpA*, AP1, AP2, *tlh*, *pirA* (AP3), *toxA*, *pirA*, *pirB*, qPCR products, and *blaCARB-17* amplification in AHPND-positive isolates. 1% agarose gel shows the amplicons of target genes in this strain. Lane M: DNA marker; lane 2–16: positive amplicons of representative strain of *Vibrio parahaemolyticus* (ahpn-25) respectively. Molecular weight, in the first and last lane, corresponds to ladder 100 bp

Fig. 2 **a** Phylogenetic tree of AHPND-positive isolates and the reference strains. AHPND-positive isolates used in this study are named in ahpn-25 as a representative strain. The neighbor-joining tree shows the phylogenetic

et al. 2015b), *trh* (Suthienkul et al. 1995), *tdh* (Karunasagar et al. 1996), *trh* (Dickinson et al. 2013) and toxic genes *toxR* (Neogi et al. 2010) *toxA* (Dangtip et al. 2015), and *pirB* (Han et al.

Fig. 3 Multiple sequence alignment and pairwise alignment score (right) of 16S rRNA, *atpA*, *blaCARB-17*, *pirA* (AP3), *tdh*, *tlh*, *tox*, and *trh* partial gene sequences with the reference strain of *Vibrio parahaemolyticus* accession no. CP034571.1 using ClustalW and DNAMAN

2015b) were isolated from 23 AHPND-positive isolates. AHPND causes early mortality syndrome (De Schryver et al. 2014) and outbreaks (Chonsin et al. 2015) in the shrimp aquaculture industry of Bangladesh. As previously reported, AHPND is characterized by two *pir* genes (Lai et al. 2015) which are deadly (Lee et al. 2015), binary toxins *pirA* and *pirB* (Kondo et al. 2014; Han et al. 2015b) genes found in isolates of *V. parahaemolyticus* of *P. monodon*.

To construct the phylogenetic relationship (Li et al. 2016), 8 *Vibrio* spp. were selected from GeneBank to compare with target gene sequences; all genes were derived from a common ancestor. The derived traits of *pirA*, *atpA*, 16S rRNA, *tox*, and *tdh* genes had a common ancestor sharing 100% identity with the reference strain of *V. parahaemolyticus* (accession nos. CP028145.1, AP014860.1, and KM067908.1); *trh*, *blaCARB-17*, and *tlh* genes had a common ancestor sharing 100% identity with the reference strain of *V. parahaemolyticus* (accession no. AP014859.1) (Fig. 2a). All genes showed a common ancestor (accession no. CP022245.1), wherein all clades were derived with different traits with very low genetic distance (Fig. 2b). The phylogenetic relationship in the sequence of target genes showed that AHPND-positive isolates belonged to *Vibrio* spp. and all of which were close to *V. parahaemolyticus*. The variability (Liu et al. 2015) of the target genes (*toxR*, 16S rRNA, *pirA* (AP3), *tox*, *blaCARB-17*, *tlh*, *atpA*, *tdh*, and *trh*) sequence was analyzed. The few mutations and sequence inaccuracy were identified in the starting and middle regions of the genes. The genetic variations (Chonsin et al. 2015) including missing nucleotide through gene sequencing were examined.

The sequences of the PCR amplification (Kondo et al. 2014; Restrepo et al. 2016) of target genes (*toxR*, 16S rRNA, *pirA* (AP3), *tox*, *blaCARB-17*, *tlh*, *atpA*, *tdh*, and *trh*) from an AHPND-positive representative strain (ahpn-25) were aligned with the reference strain of *V. parahaemolyticus* (accession no. CP034571.1) with low variability (Fig. 3). The *atpA* gene in AHPND-positive isolates produced high homology and 100% pairwise alignment with the reference strain in this research. The eight different target genes were aligned and obtained a consensus sequence. The pairwise alignment scores for *atpA*, *tox*, *blaCARB-17*, 16S rRNA, and *pirA* gene were 100.0, 98.90, 98.89, 95.53, and 41.42, respectively, in the AHPND-

Fig. 4 Comparison of sequence homology; the sequence of AHPND-positive strain (ahpn-25) for eight genes used as *Vibrio parahaemolyticus* detection targets. The 16S rRNA, *pirA* (AP3), *tox*, *blaCARB-17*, *tlh*, *atpA*, *tdh*, and *trh* gene homology compared with *Vibrio* sp. (NCBI accession no. LK021128.1, AP014860.1, CP021148.1, and CP033146.1)

positive strain ahpn-25. Variations included a missing nucleotide position in the sequence of all target genes in this study analyzed by ORFfinder and RCSB PDB, by using Smart BLAST (CDD) and NCB (Marchler-Bauer et al. 2017; Marchler-Bauer et al. 2015; Marchler-Bauer et al. 2013). This result revealed some bacterial virulence proteins such as VirB8 and VirB10 (accession no: cl01500), TrbE (accession no: cl36305), sopB_N and ParB (accession nos: cl16394 and cl38291), TraF (accession no: cl31246), Tra8 (accession no: COG2826),

Distance matrix of 9 sequences

AP014860.1	0							
ahpn-25_16sRNA.txt	0.653	0						
ahpn-25_atpA.txt	0.693	0.688	0					
ahpn-25_blaCARB.txt	0.700	0.681	0.682	0				
ahpn-25_trh.txt	0.736	0.724	0.689	0.684	0			
ahpn-25_pirA.txt	0.707	0.707	0.717	0.679	0.707	0		
ahpn-25_tdh.txt	0.000	0.653	0.693	0.700	0.736	0.707	0	
ahpn-25_tlh.txt	0.719	0.719	0.729	0.714	0.707	0.726	0.719	0
ahpn-25_tox.txt	0.632	0.658	0.700	0.679	0.702	0.686	0.632	0.695

Homology matrix of 9 sequences

AP014860.1	100%							
ahpn-25_16sRNA.txt	34.7%	100%						
ahpn-25_atpA.txt	30.7%	31.2%	100%					
ahpn-25_blaCARB.txt	30.0%	31.9%	31.8%	100%				
ahpn-25_trh.txt	26.4%	27.6%	31.1%	31.6%	100%			
ahpn-25_pirA.txt	29.3%	29.3%	28.3%	32.1%	29.3%	100%		
ahpn-25_tdh.txt	100.0%	34.7%	30.7%	30.0%	26.4%	29.3%	100%	
ahpn-25_tlh.txt	28.1%	28.1%	27.1%	28.6%	29.3%	27.4%	28.1%	100%
ahpn-25_tox.txt	36.8%	34.2%	30.0%	32.1%	29.8%	31.4%	36.8%	30.5%

Fig. 5 Distance and homology matrix of AHPND-positive strain (ahpn-25) for eight genes used as *Vibrio parahaemolyticus* detection targets compared with *V. parahaemolyticus* (NCBI accession no. AP014860.1)

PRK00409 (accession no: cl29770), and SR_Res par (accession no: cd03767), which may conserved the protein domain family in the sequences of 16S rRNA, *blaCARB*-17, *pirA*, *tdh*, *tlh*, *tox*, and *trh* gene.

The PCR amplification, agarose gel electrophoresis, and RT-qPCR assay of target genes 16S rRNA, *blaCARB*-17 and *atpA* revealed accurate amplified fragment in Fig. 1 and comparatively high expression and constantly maintained 100% specificity in Table 2 for *V. parahaemolyticus* (Izumiyama et al. 2011; Dickinson et al. 2013) in parallel with different isolates in Table 3. Accordingly, the PCR, electrophoresis, and RT-qPCR assay of target genes

Fig. 6 Relative gene expression levels determined by RT-qPCR of *pirA* in AHPND-positive isolates incubated for 4 h at 37 °C in tryptone soya broth (TSB) culture medium. A non-AHPND strain ATCC17802 was used as the negative control. Error bars indicate standard error

toxR, *pirA* (AP3), *toxA*, *tlh*, *tdh*, and *trh* yielded less specificity and low expression. The level of specificity is unsatisfactory in rapid molecular methods for identifying *V. parahaemolyticus*, thereby hindering its comprehensive application in routine laboratory tests (Klein et al. 2014). This result assured that the isolates isolated from shrimp farms were all toxic to shrimp, but their virulence was different, and the relative expression level was variable between isolates. In this study, the relative expression levels determined by RT-qPCR (Karunasagar et al. 1996; Gutierrez West et al. 2013) of target genes showed that nine target genes were expressed in AHPND-positive representative strain, but that levels of expression were variable. The *atpA*-like gene exhibited the lowest degrees of similarity in *V. parahaemolyticus* and highest in *V. anguillarum* via homology analysis, indicating the most divergence in AHPND-positive isolates (Restrepo et al. 2016). The homology distance matrix for target genes of AHPND-positive strain (ahpn-25) was compared with that of reference strain (accession no: AP014860.1).

The sensitivity and specificity of RT-qPCR applied in this work can be useful to detect *V. parahaemolyticus* in shrimp (*Penaeus monodon*) in epidemiological research. All target genes in *V. parahaemolyticus* that may be causal agents of AHPND in shrimp may require a suitable vaccine development to prevent AHPND in Bangladesh.

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Author contribution Dr. Md. Mer Mosharraf Hossain, Dr. Imtiaz Uddin, and Dr. Md. Anisur Rahman designed the study, and Habiba Islam, Jannatul Fardous, Md. Ariful Haque Rupom, Md. Monjur Hossain, Nawshin Farjana, Rukaiya Afroz, and Md. Asif Shahriar Shehab conducted the experiments, analyzed the data, and wrote the manuscript. Dr. Md. Mer Mosharraf Hossain was actively associated with experimental works and data processing. Hasan-Uj-Jaman and Hironmoy Shovon Roy was directly involved with shrimp farm and shrimp farmers to collect different kind of shrimp samples (healthy or diseased). The authors would like to thank everyone who was indirectly associated with this work. All authors read and approved the manuscript. Data availability The data in this article had not been previously shared or published anywhere else. The datasets supporting the conclusions of this article are included with the article.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval The ethical committee of the Department of Fisheries and Marine Bioscience, Jashore University of Science and Technology, Bangladesh, approved these experiments.

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