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MOLECULAR CHARACTERIZATION OF ACUTE HEPATOPANCREATIC NECROSIS DISEASE CAUSING *VIBRIO PARAHAEMOLYTICUS* STRAINS IN CULTURED SHRIMP *PENAEUS MONODON* IN SOUTH-WEST FARMING REGION OF BANGLADESH

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Key words: Vibrio parahaemolyticus, *Penaeus monodon*, AHPND, Shrimp disease

Abstract

Acute hepatopancreatic necrosis disease (AHPND) is an emerging shrimp disease caused by strains of *Vibrio parahaemolyticus* containing a unique virulent plasmid, responsible for substantial economic losses since 2009; caused up to 100% mortality in farmed shrimp *Penaeus monodon*. The purpose of this study was to isolate and identify the pathogenic strain of *V. parahaemolyticus* causing AHPND in cultured shrimp (*Penaeus monodon*) using classical and molecular techniques. Samples were collected from three different locations of south-west shrimp farming regions of Bangladesh *viz.* Sadar Upazilla of Satkhira; Mongla and Morrelganj under Bagerhat district. In this study, three selective media were used for primary isolation of *V. parahaemolyticus*. Among 46 primary isolates, 18 representative isolates were checked for the species-specific detection of *V. parahaemolyticus* using *ldh* primers and all of them were found to be positive. 16S rRNA gene sequencing were used to further confirm the isolates as *V. parahaemolyticus*. *tdh* primer was used to check human pathogenicity but all 18 isolates showed negative result. The isolates were further characterized to check their AHPND positivity using AP3 and AP4 primers. Ten isolates showed positive results for AP3 (55.56%) and 9 showed positive results for AP4 (50%) which indicated that the isolates were AHPND positive. This study also reported that all AHPND positive strains were resistant to the antibiotic gentamycin but sensitive to chloramphenicol, nalidixic acid, nitrofurantoin and tetracycline. The findings of this study will help the shrimp farmers and policy makers to take proper biosecurity measures to protect shrimps from AHPND and thereby sustain the shrimp production in Bangladesh.

Introduction

The shrimp aquaculture industry is continuously growing and accounts for 15% of the internationally traded seafood products(1). Farmed shrimp account for 57% of the total shrimp consumed globally (7.9 million tons)(1). China, Vietnam, Thailand and Bangladesh

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are the major shrimp producing countries[1]. Although global shrimp production has increased from approximately one million tons in 2000 to four million tons in 2011, the production level of 2015 was expected to be 15% below that of 2011 due to disease outbreaks causing significant production losses[1]. Asian shrimp farming industry has experienced massive production losses due to a disease caused by toxins of *Vibrio* bacteria, known as early mortality syndrome/acute hepatopancreatic necrosis disease (EMS/AHPND) for the last 5 years[2]. It is estimated that about 60% of disease losses in shrimp aquaculture have been caused by viral pathogens and only 20% by bacterial pathogens[3]; but this emerging disease AHPND may shift this scenario in favor of bacteria. This disease has recently caused serious production loss in the shrimp culture industry. It most frequently occurs within the first 30 days after stocking a newly prepared shrimp pond. It was first reported in China in 2009 and then spread to Vietnam, Malaysia and Thailand[4]. AHPND affects both *Penaeus monodon* (Fabricius) and *Litopenaeus vannamei* (Boone). Certain strains of *V. parahaemolyticus* belonging to the Harveyi clade are responsible for pathological changes in the hepatopancreas (HP) of AHPND affected animals[5]. *Vibrio parahaemolyticus* is a halophilic Gram-negative bacterium that is a normal inhabitant of marine environment. Most of the *V. parahaemolyticus* isolated from the marine environments are non-pathogenic strains. AHPND suspected shrimps show gross signs of abnormal HP with significant atrophy and discoloration when compared with the normal shrimps[4]. The histopathology of AHPND is characterized by massive cell sloughing of HP tubule epithelial cells together with the dysfunction of B, F, R and E cells of HP of affected shrimps[6]. Detection of *V. parahaemolyticus* isolates is typically based on molecular biological analysis that amplify species-specific gene *ldh* (lecithin dependent hemolysin)[7] and *tdh* (thermostable direct hemolysin)[8] gene is amplify to detect human pathogenicity. Among all sequencing platforms, 16S rRNA sequencing technology provides large enough length (1500 bp) for informatics purposes and has been used to investigate bacterial phylogeny and taxonomy[9,10]. AP3[11] and AP4[12] primers are usually used to characterize AHPND positive strains of *V. parahaemolyticus*.

The aim of the study was to isolate and characterize AHPND positive *V. parahaemolyticus* from shrimp farms of Bangladesh using molecular techniques. Moreover, construction of phylogenetic tree using 16S rRNA gene sequencing, human pathogenicity and antibiotic susceptibility of the isolated bacteria were also performed.

Materials and Methods

Twenty seven shrimp samples from 20 shrimp farms (locally known as *Ghers*) were collected from Sadar Upazilla of Satkhira district and two Upazilla (Morrelganj and Mongla) of Bagerhat district and immediately transported to the laboratory. To lower the temperature (4°C) during transportation, ice was placed around the bags. The shrimp

samples were processed and hepatopancreas were collected within 12 hours of collection following aseptic techniques [13].

Sampling of hepatopancreas was done carefully to avoid contamination with intestinal bacteria and serial dilutions was done in sterile saline solution. Three selective agar media *viz*., TCBS (Thiosulphate Citrate Bile Salt Sucrose Agar), Hi Chrome *Vibrio* agar and Chrom Agar *Vibrio* were used for culturing bacteria after enrichment in alkaline peptone water (APW). After incubation at 37°C, characteristic single colonies were picked up and streaked on TCBS agar and Chrom Agar *Vibrio* plates and incubated at 37°C overnight. Green and pure purple colored colonies were selected and were streaked onto Tryptic Soya Agar (TSA) with 2% salt for purity assurance. Pure isolates were preserved in LB media with 30% glycerol and frozen at –80°C for further studies.

After DNA extraction following Rahman *et al*.[14] suspected colonies were analyzed for the presence of the species-specific molecular markers of *V. parahaemolyticus*, i.e. *ldh* as described by Taniguchi *et al*.[7]. 16S rRNA gene sequencing of about 1500 bp was used for further confirmation of the isolates as *V. parahaemolyticus*. Universal primers for 16S rRNA amplification are listed in Table 1. PCR reaction mixture and thermal cycling condition were done following Punom *et al*.[15].

Amplified DNA was further purified with the Wizard PCR SV Gel and PCR Clean-Up System (Promega, USA) according to the manufacturer instruction prior to sequencing. Sequencing of PCR product was performed using Big Dye Terminator v 3.1 Cycle sequencing Kit (Applied Biosystems, USA) according to manufacturer instruction and capillary electrophoresis was done using ABI Genetic Analyzer (Applied Biosystems, USA). To view DNA sequence, Geospizas Finch TV version 1.4 was used. BLAST (Basic Local Alignment Search Tool) was used for comparing primary sequence information. MEGA v 6.0[16] was used for the comparative analysis of molecular sequence data for reconstructing the evolutionary histories of species and inferring the phylogeny by using the Maximum Likelihood method based on the Tamura-Nei model[17].

Only isolates positive for *ldh* and confirmed by 16S rRNA sequencing were further analyzed by PCR with *tdh*[8] to check the human pathogenicity. AP3 and AP4 (nested) primers were used to check the AHPND positivity as described by Sirikharin *et al*.[11] and Dangtip *et al*.[12], respectively. These primers target contigs that belong to a plasmid in AHPND positive pathogenic strains of *V. parahaemolyticus*. A list of primers to identify and characterize AHPND positive *V. parahaemolyticus* is given in Table 1.

The Kirby-Bauer disc diffusion technique[18] was performed to determine the sensitivity or resistance of pathogenic bacteria to 14 antibacterial compounds (list of 14 antibiotic discs is given in Table 4). Eighteen representative isolates were inoculated on Muller-Hinton Broth (MHB) (Oxoid, USA) and incubated for 24 hrs and then the bacterial suspension was spreaded onto the surface of the Muller-Hinton Agar (MHA) using sterile cotton swabs, which were then left to dry for several minutes. The antibiotic discs

(Oxoid, USA) were applied on the surface of the agar plate and incubated for 24 hrs at 37°C. Finally, the zone of inhibition was measured to detect susceptibility of the bacteria.

Table 1. List of primers to identify and characterize AHPND positive *V. parahaemolyticus*.

Sl.	Primers	Sequence	Purpose	References
1	*ldh*	5`-AAAGCGGATTATGCAGAAGCACTG-3` 3`-GCTACTTTCTAGCATTTTCTCTGC-5`	To detect *V. parahaemolyticus* haemolysin gene	Taniguchi *et al*.[7]
2	16S rRNA	5′-GCCTAACACATGCAAGTCGA-3′ 5′-GACTACCAGGGTATCTAATCC-3′	To further confirm *V. parahaemolyticus* isolates	Lane[9] Frank *et al*.[10]
3	*tdh*	5`-GTACCGATATTTTGCAAA-3` 3`-ATGTTGAAGCTGTACTTGA-5`	To detect the human pathogenicity	Bej *et al*.[8]
4	AP3	5′-ATGAGTAACAATATAAAACATGAAAC-3′ 5′-GTGGTAATAGATTGTACAGAA-3′	To detect *Tox*A and *Tox*B gene	Sirikharin *et al*.[11]
5	AP4-F1* AP4-R1	5`-ATGAGTAACAATATAAAACATGAAAC-3` 3`-ACGATTTCGACGTTCCCCAA-5`	To detect *Tox*A and *Tox*B gene	Dangtip *et al*.[12]
	AP4-F2 AP4-R2	5`-TTGAGAATACGGGACGTGGG-3` 3`-GTTAGTCATGTGAGCACCTTC-5`	To detect *Tox*A gene plus 12 bp spacer sequence plus 9 bp*Tox*B gene	Dangtip *et al*.[12]

*Primer AP4-F1 is identical to primer AP3-F from the AP3 method.

Nucleotide sequence accession numbers: The reported sequences in this study have been submitted to NCBI GenBank database under accession numbers KY565404-KY565421.

Results and Discussion

Three types of selective culture media were used for primary isolation of *V. parahaemolyticus*. Forty six *V. parahaemolyticus* strains were isolated from 27 shrimp samples based on colony morphology (Green Colonies on TCBS plate, Violet Colonies on Chrom Agar *Vibrio* and Bluish green Colonies on HiChrome *Vibrio* Agar).

Eighteen representative isolates (Vp2, Vp4, Vp6, Vp7, Vp9, Vp11, Vp21, Vp23, Vp24, Vp25, Vp26, Vp27, Vp30, Vp35, Vp38, Vp39, Vp42 and Vp43) were used for the detection of *V. parahaemolyticus* by using species specific *ldh* primer. Detection for *ldh* gene fragment showed positive result for all 18 representative isolates.. In a study on AHPND of shrimp, Joshi *et al*.[7] identified six bacterial isolates as *V. parahaemolyticus* by lecithin dependent hemolysin (*ldh*) gene primer.

The amplified PCR products (Fig. 1) were sequenced and aligned with the 16S rRNA gene sequences in GenBank and the sequences showed (>99%) homology with the 16S rRNA gene of *V. parahaemolyticus* strains. The identification of the 16S rRNA gene sequences of 18 representative isolates through nucleotide BLAST of NCBI is summarized on Table 2.

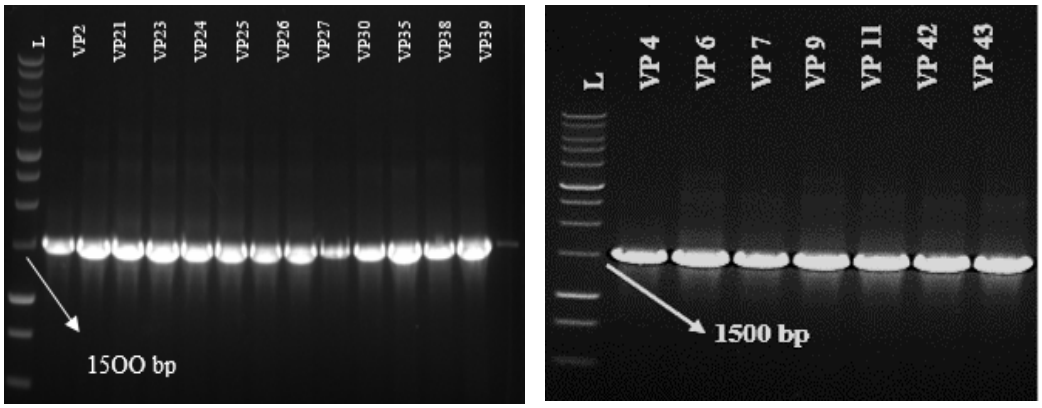

Fig. 1. PCR amplification of 16S rRNA gene of 18 representative isolates of *V. parahaemolyticus* and L denotes DNA ladder of 1kb marker.

Phylogenetic tree was constructed using 16S rRNA gene sequences of 18 representative isolates of this study and 9 sequences were downloaded from NCBI GenBank. The taxonomic position of all 18 representative isolates was confirmed as *V. parahaemolyticus* according to the phylogenetic tree (Fig. 2). The isolates clustered with various strains of *V. parahaemolyticus* rather other *Vibrio* strains. *Aliivibriofischeri* strain CZB-30 was used as an out group during phylogenetic reconstruction. In a study, Joshi *et al*.[6] also analyzed their isolates with 16S ssurRNA gene sequences and found their isolates gave 99% similarity with *V. parahaemolyticus*. Present investigation strongly suggests that the presumptive identification of the isolates through selective media was confirmed as the strains of *V. parahaemolyticus*.

Representative 18 confirmed *V. parahaemolyticus* isolates were then used for the detection of human pathogenicity by using *tdh* primer. Detection for *tdh* gene fragment showed negative results for all the isolates. Xu *et al*. [19] worked on 145 *V. parahaemolyticus* isolates to confirm and test for the presence of *tdh*. But they found that none of the isolates possessed the genes. Soto-Rodriguez *et al*.[20] confirmed 37 isolates as *V. parahaemolyticus* using *tlh* but those isolates were found negative for the human-toxigenic genes *tdh* and *trh*.

Table 2. Similarity of sequences of 16S rRNA gene of representative isolates of this study with those obtained from BLAST search.

Sl .No.	Sample ID	Sampling area	Isolates ID	Description	BLAST Score	Identity (%)	GenBank accession no.	Accession no. of our strains
1	SH1	Satkhira	Vp2	*Vibrio parahaemolyticus* strain ECSMC9	2555	99	KU845385.1	KY565404
2	AH2	Satkhira	Vp4	*Vibrio parahaemolyticus* strain CHB-35	2560	99	KR347292.1	KY565405
3	MiH3	Satkhira	Vp6	*Vibrio parahaemolyticus* strain Xmb045	2549	99	KT986171.1	KY565406
4	OH4	Satkhira	Vp7	*Vibrio parahaemolyticus* strain NSP1	2555	99	JN188415.1	KY565407
5	BH5	Satkhira	Vp9	*Vibrio parahaemolyticus* strain XG409	2549	99	JQ948037.1	KY565408
6	M2H6	Satkhira	Vp11	*Vibrio parahaemolyticus* strain M2-31	2549	99	KC210810.1	KY565409
7	AnH1	Satkhira	Vp21	*Vibrio parahaemolyticus* strain M2-11	2560	99	KC210810.1	KY565410
8	AbH2	Satkhira	Vp23	*Vibrio parahaemolyticus* strain CHB-5	2555	99	KR347274.1	KY565411
9	AbH2	Satkhira	Vp24	*Vibrio parahaemolyticus* strain Aj2010072802A90	2560	99	JF432066.1	KY565412
10	AbH2	Satkhira	Vp25	*Vibrio parahaemolyticus* strain FORC_018	2555	99	CP013826.1	KY565413
11	BaH1	Satkhira	Vp26	*Vibrio parahaemolyticus* strain L41	2555	99	KC884619.1	KY565414
12	Shw1	Satkhira	Vp27	*Vibrio parahaemolyticus* strain CZN-9	2560	100	KR347248.1	KY565415
13	AbW1	Satkhira	Vp30	*Vibrio parahaemolyticus* strain CHB-40	2555	99	KR347297.1	KY565416
14	BaHM	Bagerhat	Vp35	*Vibrio parahaemolyticus* strain CZN-34	2549	99	KR347270.1	KY565417
15	MiHB	Bagerhat	Vp38	*Vibrio parahaemolyticus* strain CZN-9	2555	99	KR347248.1	KY565418
16	MeHB	Bagerhat	Vp39	*Vibrio parahaemolyticus* strain CHB-33	2549	99	KR347290.1	KY565419
17	AfHB	Bagerhat	Vp42	*Vibrio parahaemolyticus* strain NSTH21	2549	99	KF886632.1	KY565420
18	SeHB	Bagerhat	Vp43	*Vibrio parahaemolyticus* strain CZN-7	2555	99	KR347246.1	KY565421

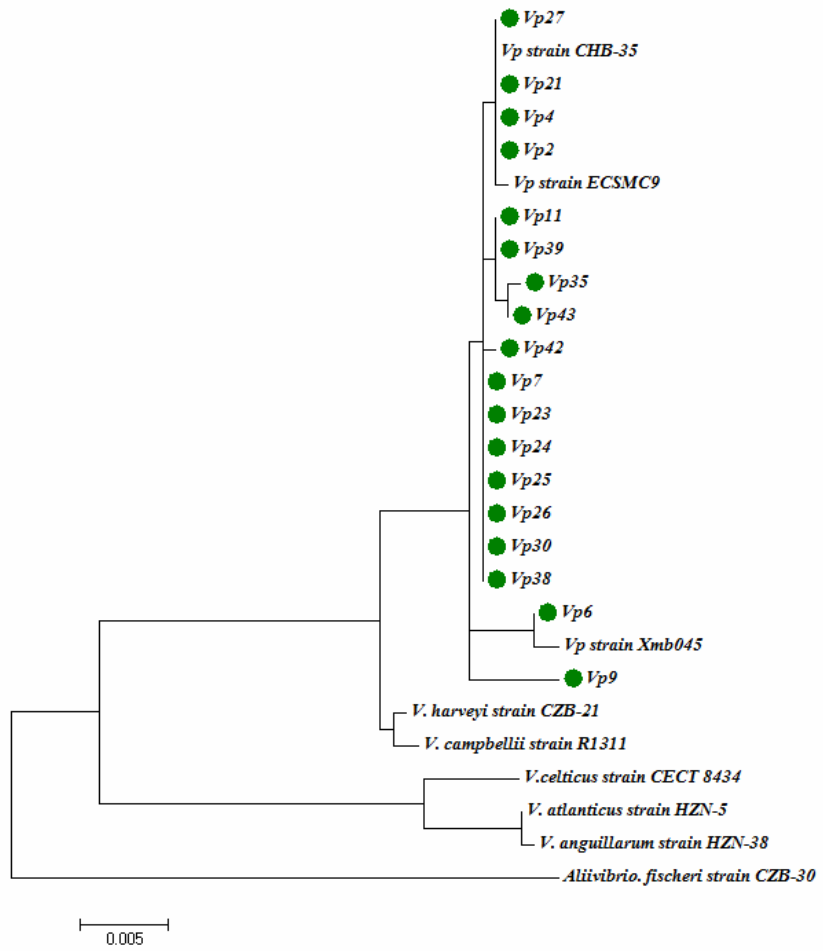

Fig. 2. Molecular phylogenetic analysis of 18 representative *V. parahaemolyticus* isolates by Maximum Likelihood method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 1384 positions in the final data set. Evolutionary analyses were conducted in MEGA[16]. ● indicates *V. parahaemolyticus* isolates of this study.

The AP3 primers are specific for *V. parahaemolyticus* strains causing AHPND. In the present study, out of 18 representative isolates ten were found positive for AP3 specific primer (Fig. 3) and eight isolates were negative (Table 3). Kongrueng *et al*.[21] used AP3 that targeted to the unique DNA sequences of toxin gene of AHPND positive *V. parahaemolyticus* and found 33 positive isolates from shrimp, whereas they found negative result for all clinical and environmental isolates. In another study, Soto-Rodriguez *et al*.[20] also used AP3 primer for the detection of pathogenic and nonpathogenic *V. parahaemolyticus* strains. However, AP3 showed a false-positive result in that study (strain M06-04) and produced a predictive positive value of 90%.

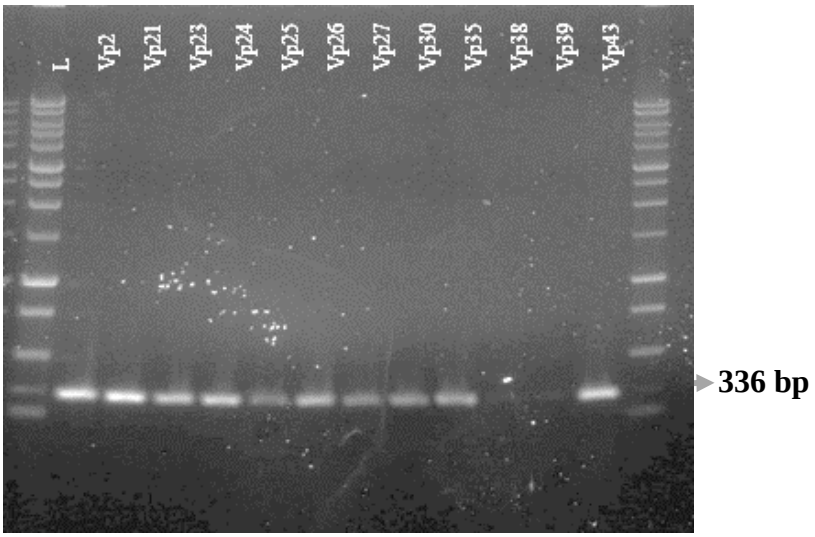

Fig. 3. PCR amplification with the primer AP3 for *V. parahaemolyticus* isolates of shrimp collected from south-west regions of Bangladesh. L denotes DNA ladder of 1kb marker.

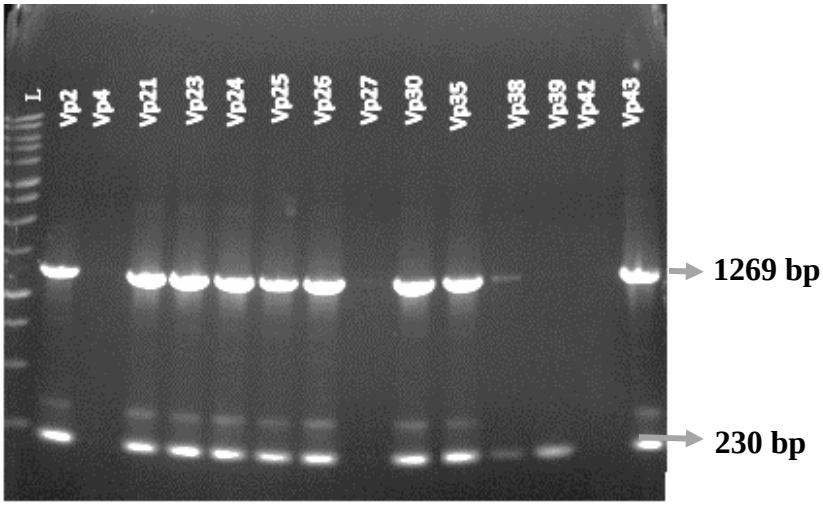

Fig. 4. Agarose gel electrophoresis of PCR amplified DNA fragments from representative *V. parahaemolyticus* isolates using AP4 nested PCR. L denotes DNA ladder of 1kb marker.

The same 18 representative isolates were also subjected to PCR using AHPND specific nested primer pairs AP4. A total of the 9 isolates of *V. parahaemolyticus* were found positive (Fig. 4, Table 3). The isolates that gave positive result with AP3, same isolates gave positive result with AP4 except for Vp27.Dangtip *et al.* [12] worked with AP4 primers and found 51 AHPND positive *V. parahaemolyticus* out of 104 bacterial isolates.

All representative 18 isolates of *V. parahaemolyticus* were resistant to the antibiotic gentamycin whereas all isolates showed 100% sensitivityto chloramphenicol, nitrofurantoin, tetracycline and nalidixic acid (Table 4). Among 18 isolates of *V. parahaemolyticus*, 15 isolates were intermediate and 3 showed sensitivity to Ciprofloxacin. On the other hand, 16 isolates showed Amikacin sensitivity and 2 isolates were intermediate but no resistant isolate was found. All isolates of *Vibrio parahaemolyticus* showed various level of sensitivity pattern to ampicillin, erythromycin, streptomycin and sulphamethoxazole. De Melo *et al*.[22] and Xu *et al*.[19] reported antibiotic susceptibility of *V. parahaemolyticus* isolates from shrimp and other aquatic products and found various levels of sensitivity against seven and 12 antimicrobial agents, respectively. Their studies indicate considerable risk of development of antibiotic resistance throughout the food chain.

Table 3. Detection of AHPND positive *V. parahaemolyticus* isolates using AP3 and AP4 primer based PCR assay.

Isolate name	Region	AP3 primer based PCR assay	AP4 primer based PCR Assay
Vp2	Satkhira	+	+
Vp4	"	-	-
Vp6	"	-	-
Vp7	"	-	-
Vp9	"	-	-
Vp11	"	-	-
Vp21	"	+	+
Vp23	"	+	+
Vp24	"	+	+
Vp25	"	+	+
Vp26	"	+	+
Vp27	"	+	-
Vp30	"	+	+
Vp35	Bagerhat	+	+
Vp38	"	-	-
Vp39	"	-	-
Vp42	"	-	-
Vp43	"	+	+

Table 4. Percentage of antibiotic susceptibility for 18 *V. parahaemolyticus* isolates against 14 antibiotics.

Name of Antibiotics	Isolates of bacteria (N = 18)		
	R (%)	I (%)	S (%)
Amikacin (AK) (30 μg)	0	11.11	88.89
Amoxycillin (AML) (10 μg)	88.89	5.56	5.56
Ampicillin (AMP) (10 μg)	44.45	0	55.55
Chloramphenicol (C) (30 μg)	0	0	100
Ciprofloxacin (CIP) (5 μg)	0	16.67	83.33
Erythromycin (E) (15 μg)	38.89	5.55	55.55
Gentamycin (CN) (10 μg)	100	0	0
Kanamycin (K) (30 μg)	0	44.45	55.55
Nalidixic acid (NA) (30 μg)	0	0	100
Nitrofurantoin (F) (300 unit)	0	0	100
Polymyxin B (PB) (300 unit)	11.11	0	88.89
Streptomycin (S) (10 μg)	11.11	44.45	44.45
Sulphamethoxazole (SXT) (25 μg)	22.22	44.45	33.33
Tetracycline (TE) (30 μg)	0	0	100

*R = Resistant, S = Sensitive, I = Intermediate.

In this study,*V. parahaemolyticus*, the causative agent of AHPND, was primarily identified from shrimp farming regions of Bangladesh using molecular approaches (*ldh* gene PCR, 16S rRNA sequencing, AP3 and AP4 nested PCR assays). The presence of positive isolates indicates the possibility of future outbreaks of AHPND in Bangladesh. Regular monitoring and effective bio-security measures should be necessary to save the shrimps from emerging disease outbreaks and to prevent huge economic loss from shrimp sector.

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