*J. Bangladesh Acad. Sci., Vol. 41, No. 2, 127-135, 2017*

# MOLECULAR IDENTIFICATION OF AHPND POSITIVE *VIBRIO PARAHAEMOLYTICUS* CAUSING AN OUTBREAK IN SOUTH-WEST SHRIMP FARMING REGIONS OF BANGLADESH

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## ABSTRACT

Acute hepatopancreatic necrosis disease (AHPND) of shrimps is a bacterial disease, first appeared in China in 2009 and causes mortality up to 100 % which usually occurs early (within approximately first 35 days) after stocking shrimp fry of black tiger shrimp, *Penaeus monodon* (Fabricius) and white leg shrimp, *Litopenaeus vannamei* (Boone). The purpose of this study was to isolate and identify the pathogenic strain of *V. parahaemolyticus* causing AHPND in cultured shrimps (*P. monodon*) using molecular techniques. After a disease outbreak in April 2017, shrimp samples were collected from three different locations of south-west shrimp farming region of Bangladesh *viz.* Satkhira, Khulna and Bagerhat districts. In this study, three selective media were used for primary isolation of *V. parahaemolyticus*. Among 28 primary isolates, representative14 isolates were checked for the species-specific detection of *V. parahaemolyticus* using *ldh* primers and all of them were found to be positive. The isolates were further characterized to check their AHPND positivity using AP3 and AP4 primers. Four isolates showed positive results for both AP3 and AP4 which indicated that the isolates were AHPND positive. This study also report that all AHPND positive strains showed sensitivity to the antibiotics Tetracycline and Nalidixic Acid. The results of this study will help the farmers and policy makers to make plan to protect shrimps from AHPND and thereby sustain the shrimp farming in Bangladesh.

Key words: AHPND, EMS outbreak, *V. parahaemolyticus*, *P. monodon*, Antibiogram

## INTRODUCTION

Asian shrimp cultivation is an important industry that generates billions of US dollar in export income annually (Flegel 2012). The main cultivated species are *Penaeus monodon* and *Litopenaeus vannamei*, with the latter currently dominating the world market (FAO 2013). Acute hepatopancreatic necrosis disease (AHPND) of shrimps is a bacterial disease, first appeared in China in 2009. Since then, AHPND has caused serious drops in shrimp production (up to 20 % worldwide) (Hong *et al*. 2015). AHPND has caused mortality up to 100 % which usually occurs early (within approximately 35 days)

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after stocking shrimp fry in shrimp ponds; therefore, it was initially referred to as early mortality syndrome (EMS). AHPND affects black tiger shrimp, *Penaeus monodon* (Fabricius) and white leg shrimp, *Litopenaeus vannamei* (Boone). The scientists recently found that EMS/AHPND could be initiated by a bacterial agent termed *V. parahaemolyticus* which is transferred through oral and then localizes in the shrimp gastrointestinal tract and create a poison that causes tissue devastation and invalidism of the shrimp digestive system known as the hepatopancreas. Tran *et al.* (2013) showed that certain strains of *V. parahaemolyticus* belonging to the Harveyi clade are responsible for pathological changes in the hepatopancreas of EMS/AHPND affected animals and are negative for genes encoding the thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) that are associated with human pathogenic strains. *Vibrio parahaemolyticus* is a halophilic Gram-negative bacterium that is a normal inhabitant of marine environments. 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 (NACA 2012). 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 (Flegel 2012; Joshi *et al*. 2014). Detection of *V. parahaemolyticus* isolates is typically based on molecular biological analysis that amplify species-specific gene *ldh* (lecithin dependent hemolysin) (Taniguchi *et al*. 1985); and to identify the AHPND positive *V. parahaemolyticus* strains AP3 (Sirikharin *et al*. 2014) and AP4 (Dangtip *et al*. 2015) primers are commonly used.

Recently, in April 2017, there were several reports in the daily newspapers on disease outbreak in shrimp farming regions of Bagerhat and Satkhira (http://www.bd-pratidin.com/home/printnews/226104;http://shomoyerkhobor.com/article/11302). Historically in Bangladesh, black tiger shrimp were infected with White spot syndrome virus (WSSV) (Hossain *et al.* 2014); but according to those reports, this year outbreaks did not seem to be caused by WSSV. Therefore, the aim of this study was to isolate and identify the causative agent of this disease outbreak in shrimp using molecular technique. Moreover antibiotic susceptibility of the isolated *V. parahaemolyticus* was also performed.

## MATERIALS AND METHODS

In the present study, 15 shrimp samples suspected with AHPND were collected from Sadar Upazilla of Satkhira, Rampal of Bagerhat and Rupsa of Khulna Districts; and immediately transported to the Aquatic laboratory of the Department of Fisheries, University of Dhaka. Ice was placed around the bags to lower the temperature (4°C) during transportation. The shrimp samples were processed and hepatopancreas were collected within 12 hours of collection following aseptic techniques (APHA 1998).

Sampling of the hepatopancreas was done carefully to avoid contamination with intestinal bacteria. TCBS (Thiosulphate Citrate Bile Salt Sucrose Agar), Hi Chrome *Vibrio* agar, ChromAgar *Vibrio* were used for culturing of bacteria. At first, 1mL sample and 9mL alkaline peptone water (APW) were mixed and 100 µl sample was spreaded in TCBS and Hi Chrome *Vibrio* agar plate. The green colonies from the TCBS agar and the bluish green colonies from the HiChrome Agar *Vibrio* plates were further purified in TCBS, CHROMagar Vibrio and tryptic soy agar (TSA) supplemented with 2.0% NaCl. The plates were incubated at 37°C for 18 to 24 h, and selected pure isolates were then cryopreserved at -80°C in Luria Bertani (LB) with 30% glycerol.

After DNA extraction (Rahman *et al*. 2014), suspected colonies were analyzed for the presence of the species-specific molecular markers of *V. parahaemolyticus*, i.e. *ldh* (lecithin dependent hemolysin) as described by Taniguchi *et al*. (1985). AP3 and AP4 primers were also used to check the species specific pathogenicity as described by Sirikharin *et al*. (2014) and Dangtip *et al*. (2015), respectively; these primers target contigs that belong to a plasmid in pathogen. A list of primers to identify AHPND positive *V. parahaemolyticus* is given in Table 1.

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

| SL | Name of primer | Sequence | Purpose | Ref. |
|---|---|---|---|---|
| 1. | *ldh* (lecithin dependent hemolysin) | 5`-AAAGCGGATTATGCAGAA GCACTG-3`<br>3`-GCTACTTTCTAGCATTTTC TCTGC-5` | To detect *V. parahaemolyticus* haemolysin gene | Taniguchi *et al*. (1985); Joshi *et al*. (2014) |
| 2. | AP3 | 5´-ATGAGTAACAATATAAAA CATGAAAC-3´<br>5´-GTGGTAATAGATTGTACA GAA-3´ | To detect *Tox*A & *Tox*B gene | Sirikharin *et al*. (2014) |
| 3. | AP4-F1*<br>AP4-R1 | 5`-ATGAGTAACAATATAAA ACATGAAAC-3`<br>3`-ACGATTTCGACGTTCCC CAA-5` | To detect *Tox*A and *Tox*B gene | Dangtip *et al*. (2015) |
| 4. | AP4-F2<br>AP4-R2 | 5`-TTGAGAATACGGGACGTG GG-3`<br>3`-GTTAGTCATGTGAGCACC TTC-5` | To detect *Tox*A gene plus 12 bp spacer sequence plus 9bp *Tox*B gene | Dangtip *et al*. (2015) |

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

The Kirby-Bauer disc diffusion technique (Bauer *et al.* 1966) was performed to determine the sensitivity or resistance of pathogenic bacteria to 18 antibacterial compounds (list of 18 antibiotic discs is given in Table 3). Fourteen representative including 4 AHPND positive isolates were inoculated on Muller Hinton Broth (Hi-Media, M173-500G, India) and incubated for 24 hours and then the bacterial suspension

was spread onto the surface of the Muller-Hinton agar 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 hours at 37°C. Finally, the zone of inhibition was measured to detect susceptibility of the bacteria.

## RESULTS AND DISCUSSION

In this study, three types of selective culture media were used for primary isolation of *V. parahaemolyticus*. Twenty eight *V. parahaemolyticus* isolates were obtained from 15 shrimp samples based on colony morphology (Green Colonies on TCBS plate, Violet Colonies on ChromeAgar *Vibrio* and Bluish green Colonies on HiChrome *Vibrio* Agar).

Fourteen representative isolates were used for the species-specific detection of *V. parahaemolyticus* by using *ldh* primer. Detection for *ldh* gene fragment showed positive result for all isolates (Fig.1). In one study on AHPND, Joshi *et al.* (2014) also used *ldh* for the species-specific detection of *V. parahaemolyticus* and found positive PCR results with all 6 isolates.

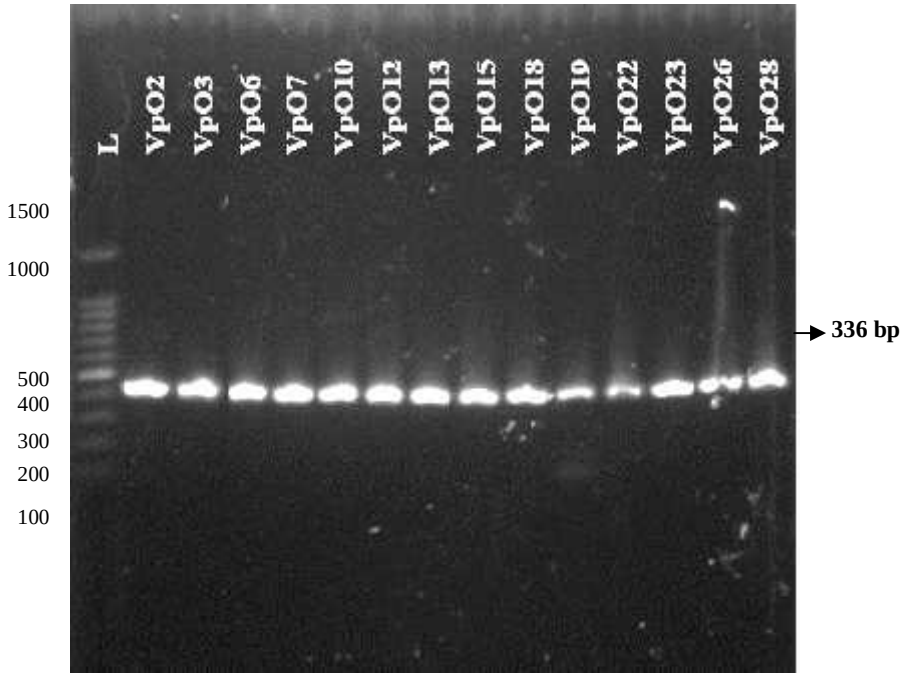


Fig 1. PCR Product profiles of *ldh* primer generated from 14 representative *V. parahaemolyticus* isolates: VpO2, VpO3, VpO6, VpO7, VpO10, VpO12, VpO13, VpO15, VpO18, VpO19, VpO22, VpO23, VpO26 and VpO28 and L denotes DNA ladder of 100 bp.

The AP3 primers are specific for *V. parahaemolyticus* strains causing AHPND. Fig. 2 illustrates that out of 14 isolates, four isolates (VpO15, VpO18, VpO22 and VpO26) were positive for AP3 specific primers.

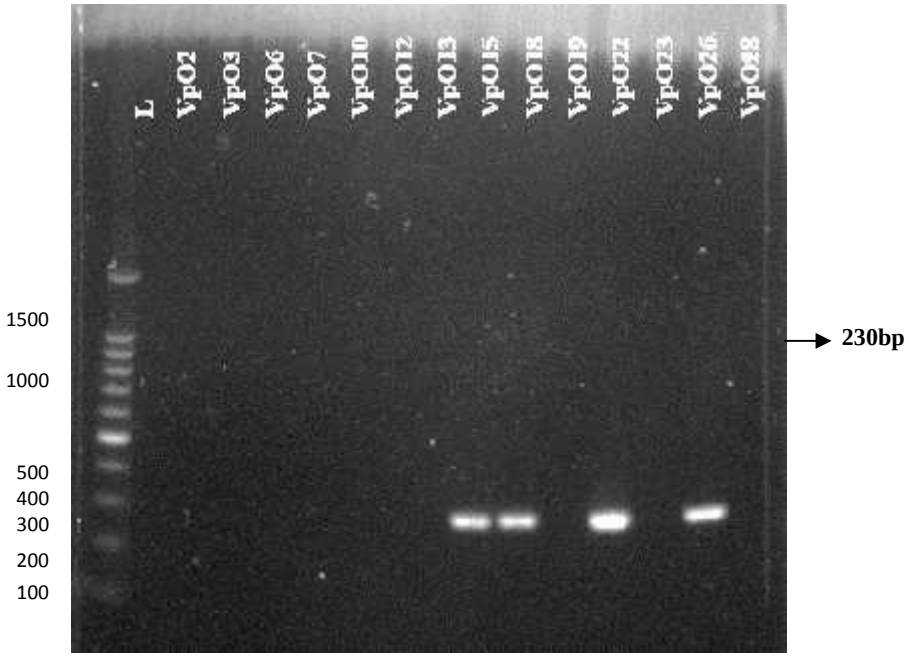


Fig 2. PCR results with the primer AP3 for representative 14 *V. parahaemolyticus* isolates of shrimp collected from southwest regions of Bangladesh. L denotes DNA ladder of 100 bp Marker.

Kongrueng *et al*. (2014) used a PCR method targeted to the unique DNA sequences derived from the plasmid (AP2 primers) and the toxin gene (AP3 primers) of *V. parahaemolyticus* that caused AHPND and found a total of 33 of 108 isolates were positive.

In the same study, they found negative results for all clinical and environmental strains of *V. parahaemolyticus* using AP3 primers. Soto-Rodriguez *et al*. (2015) also used AP3 primer for the detection of pathogenic and nonpathogenic *V. parahaemolyticus* strains. However, AP3 showed a false-positive result in this study (strain M06-04) and produced a predictive positive value of 90%.

The same 14 representative isolates were also subjected to PCR using AHPND specific nested primer pairs AP4. A total of the 4 *V. parahaemolyticus* isolates (VpO15, VpO18, VpO22 and VpO26) were found positive (Fig. 3; Table 2). Dangtip *et al.* (2015) worked with AP4 primer and they found that out of 104 isolates, 51 were AHPND positive and 53 were others bacteria including *Vibrio*.

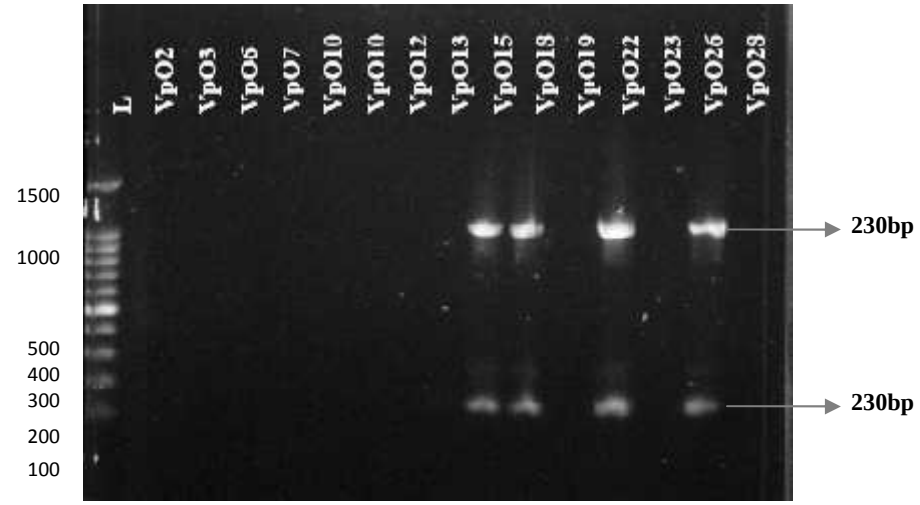


Fig 3. Agarose gel electrophoresis of PCR amplified DNA fragments from representative *V. parahaemolyticus* isolates using AP4 nested PCR. L denotes DNA ladder of 100 bp Marker.

**Table 2. Detection of $VP_{AHPND}$ isolates using AP3 and AP4 primer based PCR assay.**

| Isolate Name | Region | AP3 primer based PCR assay | AP4 primer based PCR Assay |
|---|---|---|---|
| VpO2 | Bagerhat | - | - |
| VpO3 | Bagerhat | - | - |
| VpO6 | Bagerhat | - | - |
| VpO7 | Khulna | - | - |
| VpO10 | Khulna | - | - |
| VpO12 | Khulna | - | - |
| VpO13 | Khulna | - | - |
| VpO15 | Bagerhat | + | + |
| VpO18 | Bagerhat | + | + |
| VpO19 | Satkhira | - | - |
| VpO22 | Satkhira | + | + |
| VpO23 | Satkhira | - | - |
| VpO26 | Satkhira | + | + |
| VpO28 | Satkhira | - | - |

Result of antibiotic susceptibility tests are shown in Tables 3 and 4. Representative 14 *V. parahaemolyticus* isolates showed 100% sensitivity to Tetracycline and Nalidixic Acid. On the other hand, most of the isolates were resistant to Amoxycillin (10µg) and Ampicillin (10µg). Chloramphenicol, Ciprofloxacin, Erythromycin were found to have various level of sensitivity for all isolates of *V. parahaemolyticus*. No isolates were resistant to Kanamycin, Gentamycin, Nalidixic acid and Nitrofurantoin. De Melo *et al.* (2011) reported that in antibiotic susceptibility test five strains (50%) presented multiple

antibiotic resistant to ampicillin (90%) and Amikacin (60%), while two strains (20%) displayed intermediate-level of resistance to Amikacin. Xu *et al.* (2016) reported the antimicrobial resistance patterns of 145 isolates of *V. parahaemolyticus* to 12 antimicrobial agents revealed that most of the isolates resistant to streptomycin, with resistance and intermediate rates of 86.2 % and 11.7 %, respectively. In addition, the isolates exhibited relatively high resistance rates, of 49.6 %, 43.5 %, 35.9 %, and 22.1 %, for ampicillin, cefazolin, cephalothin, and kanamycin, respectively.

**Table 3. Percentage of antibiotic sensitivity of 14 *V. parahaemolyticus* isolates against 18 antibiotics**

| Name of Antibiotics | Level of sensitivity (N=14) | | |
|---|---|---|---|
| | R% | I% | S% |
| Amikacin (AK) (30µg) | 0% | 7.14 % | 92.86% |
| Amoxycillin (AML) (10µg) | 78.58% | 14.28% | 7.14% |
| Ampicillin (AMP) (10µg) | 78.58% | 7.14% | 14.28% |
| Azithromycin (AZM) (15µg) | 7.14% | 21.42% | 71.44% |
| Ceftriaxone (CRO) (30µg) | 21.42% | 21.42% | 57.16% |
| Chloramphenicol (C) (30µg) | 14.28% | 14.28% | 71.44% |
| Ciprofloxacin (CIP) (5µg) | 7.14% | 0% | 92.86% |
| Erythromycin (E) (15µg) | 21.42% | 14.28% | 64.3% |
| Gentamycin (CN) (10µg) | 0% | 14.28% | 85.72% |
| Kanamycin (K) (30µg) | 0% | 35.71% | 64.29% |
| Nalidixic acid (NA) (30µg) | 0% | 0% | 100% |
| Nitrofurantoin (F) (300unit) | 7.14% | 14.28% | 78.58% |
| Penicillin G (P) (10µg) | 57.14% | 0% | 42.86% |
| Polymyxin B (PB) (300unit) | 7.14% | 0% | 92.86% |
| Streptomycin (S) (10µg) | 14.28% | 35.72% | 50% |
| Sulphamethoxazole (SXT) (25µg) | 14.28% | 0% | 85.72% |
| Tetracycline (TE) (30µg) | 0% | 0% | 100% |
| Trimethoprim (W) (5µg) | 28.57% | 0% | 71.43% |

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

The study was conducted to check the presence of AHPND positive *V. parahaemolyticus* during disease outbreak in April 2017 in southern shrimp farming regions of Bangladesh. A total of twenty eight isolates of *Vibrio parahaemolyticus* were obtained from the shrimp samples collected from disease outbreak areas. Four isolates were confirmed as AHPND positive using AP3 and AP4 nested PCR assay. Further investigations are needed to identify the factors that triggered the disease outbreaks in shrimp farms. Regular monitoring program and necessary policy should be formulated to prevent future disease outbreaks to booster shrimp production in Bangladesh.

**Table 4. Susceptibility of 14 representative *V. parahaemolyticus* including 4 AHPND positive isolates to tested antibiotics.**

| Isolates name | Antibiotics | | |
|---|---|---|---|
| | Sensitive | Intermediate | Resistant |
| VpO2 | AK, C, E, K, NA, CIP, PB, TE, AZM, CN | F, S | AML, AMP, SXT, P, CRO, W |
| VpO3 | PB, CN, TE, C, SXT, AZM, E, AMP, CRO, W, NA, F | K,AK | S, AML, P, CIP |
| VpO6 | PB, CN, TE, C, SXT, AZM, E, K, AMP, P, W, CIP, AK, NA | S, F, CRO | AML |
| VpO7 | PB, CN, TE, C, SXT, E, F, CRO, W, CIP, AK, NA | S, K | AML, AZM, AMP, P |
| VpO10 | S, PB, CN, TE, C, SXT, AZM, K, F, P, CIP, AK, K, NA | E | AML, AMP, CRO, W |
| VpO12 | S, PB, CN, TE, SXT, F, P, W, CIP, AK, NA | C, AZM, E, K, CRO | AML, AMP |
| VpO13 | S, PB, CN, TE, C, SXT, AZM, E, F, CRO, W, CIP, NA, AK | K | AML, AMP, P |
| VpO15 | S, PB, CN, TE, AZM, F, CRO, W, CIP, AK, NA | K | AML, C, SXT, E, AMP, P |
| VpO18 | PB, CN, TE, C, SXT, AZM, E, F, CRO, W, CIP,AK, NA | S, K | AML, AMP, P |
| VpO19 | S, PB, AMC, CN, TE, C, SXT, AZM, E, K, AMP, F, P, CRO, W, CIP, NA, AK | | |
| VpO22 | S, PB, TE, SXT, E, K, F, P, CIP, AK, NA | CN, AZM, CRO | AML, C, W, AMP |
| VpO23 | PB, TE, C, SXT, AZM, K, F, P, CIP, AK, NA | CN | S, AML, E, AMP, CRO, W |
| VpO26 | PB, CN, TE, C, SXT, AZM, K, F, CRO, W, CIP, NA, AK | S, AML | E, AMP, P |
| VpO28 | S, CN, TE, SXT, E, CRO, W, CIP, K, NA, AK | AML, C, AZM, AMP, | PB, F, P |

## ACKNOWLEDGEMENTS

We thank Md. Rakibul Islam, SSO and Ariful Islam, SO, Shrimp Research Station (SRS), BFRI, Bagerhat for their assistance during sampling. We also grateful to Sheik Md Selim Al Din, Invent Technologies for his generous support during laboratory work.

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(*Received revised manuscript on 9 November, 2017*)