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Note

Identification of *Vibrio parahaemolyticus* from farmed penaeid shrimp, *Penaeus vannamei* (Boone, 1931) by multiplex PCR targeting *toxR* and *tlh* genes

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Abstract

Targeted surveillance was conducted in 37 *Penaeus vannamei* farms in Tamil Nadu, India to find out the prevalence of *Vibrio parahaemolyticus* (*Vp*) infections. Special emphasis was given to screen the isolates for acute hepatopancreatic necrosis disease (AHPND). Bacteria were isolated from haemolymph, stomach and hepatopancreas. Initial identifications were carried out using the morphological, physiological and biochemical characteristics. *Vp* isolates were further confirmed by a newly developed multiplex PCR targeting *Vp* specific *toxR* and *tlh* genes. PCR screening was carried out for AHPND causing AP1, AP2, AP3 (*pirAvp, Photorhabdus* insect-related binary toxin gene) and AP4 (*pirAvp* and *pirBvp*) genes. In addition, PCR was also performed for human pathogenic *tdh* and trh genes. The PCR results revealed that there were 26 (35.14%) isolates affirmative for *Vp* specific *toxR* and *tlh* genes and negative for AP1, AP2, AP3 and AP4 genes indicating that there was no AHPND causing Vp strain among the isolates. The isolates were also negative for anthropozoonotic *tdh* and *trh* genes. The multiplex PCR developed targeting *Vp* specific *toxR* and *tlh* genes would be a useful technique for easy identification of *Vp* strains from shrimp farms.

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Farmed penaeid shrimps are persistently exposed to different kinds of both beneficial and pathogenic microbiota in the cultured pond environment. Though most of them are harmless, prebiotic and probiotic in nature, a few of them could lead to disease manifestation under certain environmental situations. Among them, the bacteria from the family *Vibrionaceae* are globally recorded as the most significant for causing mass mortality in farmed shrimp (Ananda Raja *et al.*, 2017a, b, c). An emerging disease known as early mortality syndrome (EMS) in shrimp (*Penaeus vannamei, Penaeus monodon* and *Penaeus chinensis*), prawn (*Macrobrachium rosenbergii*) and *Artemia franciscana* renamed as acute hepatopancreatic necrosis disease [AHPND] (FAO, 2013) has been reported to cause unusually high mortality and morbidity leading to a significant loss of one billion USD in Asia-Pacific region (FAO, 2013; Tran *et al.*, 2013). Initially, the causative agent lingered perplexing but later it has been recognised and recorded to be caused by *Vibrio parahaemolyticus* (*Vp*), *V. punensis, V. harveyi, V. owensii, V. campbellii* and *Shewanella* sp. that contains pVA1 plasmid (63-70 kb) encoding the binary PirAVP and PirBVP toxins (Kumar *et al.*, 2021). Because of outbreaks of AHPND, there are many countries neither imported live shrimp nor did any forms of shrimp products from AHPND affected

countries (FAO, 2013). Considering the impact on the global market, the current study was undertaken to isolate and identify *Vp* from *P. vannamei* farms in Tamil Nadu, India with special reference to AHPND. Multiplex PCR was developed targeting *Vp* specific *toxR* and *tlh* genes to identify the prevalence of *Vp* among the cultured *P. vannamei.*

Active surveillance against *vibriosis* was carried out in a total of 37 shrimp farms from the coastal districts, Kancheepuram and Thiruvallur of Tamil Nadu State, India between August and February. Bacteria were isolated from infected haemolymph (moribund and weak animals) based on the dominant five colonies observed on thiosulphate citrate bile salts sucrose (TCBS) plates (Zhou *et al.,* 2012). The stomach and hepatopancreas from representative shrimp ponds with mass mortality were enriched in tryptone soya broth (TSB) for 12 h and inoculated on TCBS plates for next level of bacterial characterisations (Antonio *et al.,* 2015). Thus, 74 well-separated colonies from farms showed mass mortality were selected and identified based on the species-specific characteristics (Alsina and Blanch, 1994; Ananda Raja *et al.,* 2017a, c). Vibriosis was considered as systemic when similar species of isolates were identified from both haemolymph and gut (Ananda Raja *et al.,* 2017a, b,c, Ananda Raja *et al.,* 2021). All the bacterial isolates were preserved at -20 and -80°C in duplicates in TSB with 25% glycerol (v/v).

The glycerol preserved isolates were revived in TSB and genomic DNAs of all isolates were individually mined using the standard protocol of phenol-chloroform precipitation (Hossain *et al.,* 2013; Ananda Raja *et al.,* 2017b). DNA samples were quantified using Nanodrop and tested for integrity by agarose gel (1%) electrophoresis. The samples were maintained at -80°C for future analysis. Polymerase chain reaction (PCR) was accomplished in a gradient thermal cycler (Eppendorf, HiMedia) for specific genes such as *toxR, tdh, tlh, trh,* AP1, AP2, AP3 (*pirAvp*) and AP4 (*pirAvp* and *pirBvp*) genes with PCR mixture as mentioned in Table 1. The respective gene primers, expected amplicons and references were as shown in Table 2 (Ananda Raja *et al.,* 2017b). PCR was performed with slight modification in the protocol from the cited references wherever necessary (Ananda Raja *et al.,* 2017b). Multiplex PCR was developed targeting the *Vp* specific *toxR* and *tlh* genes (Table 3). The sensitivity of PCR was checked by using serially diluted DNA samples. The PCR amplified products were examined by agarose gel electrophoresis and eluted using GenElute™ Gel Extraction Kit (Sigma) as per the manufacturer's instructions. Elutes were sequenced by a method called di-deoxy chain termination. The obtained nucleotide sequence was assembled with the help of Auto assembler (ABI Prism, USA) software. To identify the isolate with the maximum percentage of homology, BLAST analysis was carried out. The bacterial sequences were published and available in the National Center for Biotechnology Information (NCBI).

Table 1. PCR mixture for *toxR, tdh, tlh, trh,* AP1, AP2, AP3 (*pirA^{vp}*) and AP4 (*pirA^{vp} and pirB^{vp}*) genes

Components	Multiplex for *toxR* and *tlh* (µl)	Multiplex for *tdh* and *trh* (µl)	AP1 (µl)	AP2 (µl)	AP3 (µl)	AP4-I step (µl)	AP4-II step nested (µl)
Buffer without $MgCl_2$ (10x)	2.5	2.5	2.5	2.5	2.5	2.5	2.5
$MgCl_2$ (1.5 mM)	1.5	1.5	1.5	1.5	0.7 (50 mM)	0.7 (50 mM)	1.5 (50 mM)
dNTPs (200 µM)	2.0	2.0	2.0	2.0	0.4 (10 mM)	0.5 (10 mM)	0.5 (10 mM)
Taq polymerase (5 U $µl^{-1}$)	0.5	0.5	0.5	0.5	0.3	0.3	0.3
Forward primer (10 pmol)	1.0 + 1.0	1.0 + 1.0	1.0	1.0	0.5 (10 µM)	0.5 (10 µM)	0.375 (10 µM)
Reverse primer (10 pmol)	1.0 + 1.0	1.0 + 1.0	1.0	1.0	0.5 (10 µM)	0.5 (10 µM)	0.375 (10 µM)
Bacterial DNA	1.0 (1 ng)	1.0 (1 ng)	1.0 (1 ng)	1.0 (1 ng)	2.0 (100 ng)	2.0 (100 ng)	2.0 (AP4-I)
Total volume with nuclease free distilled water	15.0	15.0	15.0	15.0	25.0	25.0	25.0

Table 2. PCR gene specific primers for *toxR*, *tdh*, *tlh*, *trh*, AP1, AP2, AP3 (*pirA^{vp}*) and AP4 (*pirA^{vp}* and *pirB^{vp}*) genes

Primers	Target genes	Product size (bp)	References
*toxR*F 5′ GTCTTCTGACGCAATCGTTG 3′	*toxR*	368	Kim *et al.* (1999)
*toxR*R 5′ ATACGAGTGGTTGCTGTCATG 3′			
*tlh*F 5′ ACTCAACACAAGAAGAGATCGACAA 3′	*tlh*	208	Nordstrom *et al.* (2007)
*tlh*R 5′ GATGAGCGGTTGATGTCCAA 3′			
*tdh*F 5′ GTAAAGGTCTCTGACTTTTGGAC 3′	*tdh*	269	Bej *et al.* (1999)
*tdh*R 5′ TGGAATAGAACCTTCATCTTCACC 3′			
*trh*F 5′ TTGGCTTCGATATTTTCAGTATCT 3′	*trh*	500	
*trh*R 5′ CATAACAAACATATGCCCATTTCCG 3′			
AP1F 5′ CCTTGGGTGTGCTTAGAGGATG 3′	AP1	700	Flegel and Lo (2014)
AP1R 5′ GCAAACTATCGCGCAGAACACC 3′			
AP2F 5′ TCACCCGAATGCTCGCTTGTGG 3′	AP2	700	
AP2R 5′ CGTCGCTACTGTCTAGCTGAAG 3′			
AP3F 5′ ATGAGTAACAATATAAAACATGAAAC 3′	AP3 (*pirA^{vp}*)	333	Dangtip *et al.* (2015)
AP3R 5′ GTGGTAATAGATTGTACAGAA 3′			
AP4F1 5′ ATGAGTAACAATATAAAACATGAAAC 3′	AP4 (*pirA^{vp}* and *pirB^{vp}*)	1269	
AP4R1 5′ ACGATTTCGACGTTCCCCAA 3′			
AP4F2 5′ TTGAGAATACGGGACGTGGG 3′		230	
AP4R2 5′ GTTAGTCATGTGAGCACCTTC 3′			

Table 3. PCR condition for *toxR*, *tdh*, *tlh*, *trh*, AP1, AP2, AP3 (*pirA*^{vp}) and AP4 (*pirA*^{vp} and *pirB*^{vp}) genes

Conditions	Multiplex for *toxR* and *tlh*	Multiplex for *tdh* and *trh*	AP1 & AP2	AP3	AP4-step 1	AP4-step 2 (nested)
Initial denaturation	94°C for 5 m	94°C for 3 m	94°C for 5 m	94°C for 5 m	94°C for 2 m	94°C for 2 m
Denaturation	94°C for 30 s	94°C for 60 s	94°C for 30 s	94°C for 30 s	94°C for 30 s	94°C for 20 s
Annealing	58.4°C for 30 s	58°C for 60 s	60°C for 30 s	53°C for 30 s	55°C for 30 s	55°C for 20 s
Extension	72°C for 60 s	72°C for 60 s	72°C for 60 s	72°C for 40 s	72°C for 90 s	72°C for 20 s
No. of cycles			30			25
Final extension	72°C for 5 m	72°C for 5 m	72°C for 10 m	72°C for 5 m	72°C for 2 m	72°C for 2 m

The bacterial isolates were tested for MIC using sterile Mueller Hinton agar (MHA) plates with a depth of about 4 mm as per Bauer-Kirby method (Bauer *et al.,* 1966). The standardised bacterial suspension (0.13 OD at 625 nm) was swabbed on to MHA plates with the help of sterile non-toxic cotton swabs. The inoculated MHA plates were then left to dry for 10 min before placing the HiComb strip (Ananda Raja *et al.,* 2017a). The strips containing the following 25 antibacterial agents such as amikacin (AK), gentamicin (GEN), kanamycin (KM), neomycin (NM), streptomycin (STM), ampicillin (AMP), amoxycillin (AMX), amoxyclav (AMC), benzyl penicillin (P), cephalexin (CEX), cephotaxime (CTX), ciprofloxacin (CIPX), nalidixic acid (NA), norfloxacin (NX), ofloxacin (OFLX), vancomycin (VAN), chloramphenicol (CAP), erythromycin (ERY), mupirocin (MU), nitrofurantoin (NIT), rifampicin (RIF), sulphafurazole (SIX), trimethoprim (TMP), co-trimoxazole (COT) and tetracycline (TCN) were positioned on the individual plates and incubated at 30°C and examined after 18 h. The diameter of the zone of inhibition was calculated and equated with zone diameter interpretative chart (HiMedia, India) as per Clinical and Laboratory Standard Institute (CLSI) provided by the manufacturer.

Vp isolates were predominant (35.14%) among *P. vannamei* samples, followed by major species such as *V. harveyi* (21.62%), *V. anguillarum* (16.22%) and *V. campbellii* (10.81%). Other species such as *V. mimicus* (8.11%), *V. alginolyticus* (5.41%) and *Pseudomonas aeruginosa* (2.7%) were also observed at lesser percentage. These results were in agreement with the findings of Sudha *et al.* (2014) and Alsina and Blanch, (1994). In contrarily, Rosalind George (2002) identified *V. alginolyticus* (41.73%) as the most abundant species found in *P. monodon* culture pond water. *Vp* isolates were tested sensitive to heating. The numbers of bacterial cells were found to be at non-detectable levels when incubated at 55°C for 5 min. In addition, *Vp* did not grow in peptone water at 4°C for 18 h and not survived at −18 to −20°C after 30 days of incubation (Zorriehzahra and Banaederakhshan, 2015). Hence, the transmission of V_p could be prevented either by heating or freezing the shrimp products to the desired temperature.

Out of 74 isolates, twenty-six (35.14%) isolates were positive for *Vp* specific *toxR* and *tlh* genes in the multiplex PCR (Fig. 1) [Kim *et al.,* 1999; Nordstrom *et al.,* 2007]. All the isolates were negative by the PCR screening for the presence of AHPND causing genes among the collected farmed shrimp samples (Flegel and Lo, 2014; Dangtip *et al.,* 2015) as well as different gradient PCR procedures (Ananda Raja *et al.,* 2017b). All the seventy-four isolates were also recognised to be negative for *trh* and *tdh* genes (Bej *et al.,* 1999; Ananda Raja *et al.,* 2017b).

It has been challenging to differentiate virulent Vp bacterial strains from avirulent strains with the help of growth phenotypes *i.e.,* traditional culture methods (Takahashi *et al.,* 2005). So, PCR assays were becoming increasingly popular for detection of pathogenic bacterial strains targeting virulent genes (Hossain *et al.,* 2012). Compared to the conventional microbiological culture methods, this multiplex PCR was found to be quick and steadfast for detection of pathogenic *Vp* in shrimp. Silvester *et al.* (2015) confirmed 75 isolates as *Vp* using a PCR assay targeting the species-specific *tlh* gene and found that the prevalence of *Vp* was 71.6% in the Cochin Estuary and 53.3% in the shrimp farms. Letchumanan *et al.* (2015) indicated that a total of 57.8 and 44.4% isolates were positive for *VP* with *toxR*-based PCR assay. *Vp* prevalence in the present study was lower (35.14%) when compared to that of the earlier reports (Letchumanan *et al.,* 2015). The percentage of sequence homologies of rRNA (Kita-Tsukamoto *et al.,* 1993) and *gyrB* (Venkateswaran *et al.,* 1998) was reported to be more than 99 and 86.8% among *Vp* and *V. alginolyticus*, correspondingly. Hence, *toxR* was recognised as a gene identical to *Vp.* The *toxR* was also renowned for the regulation of several genes in *V. cholerae* (DiRita, 1992). However,

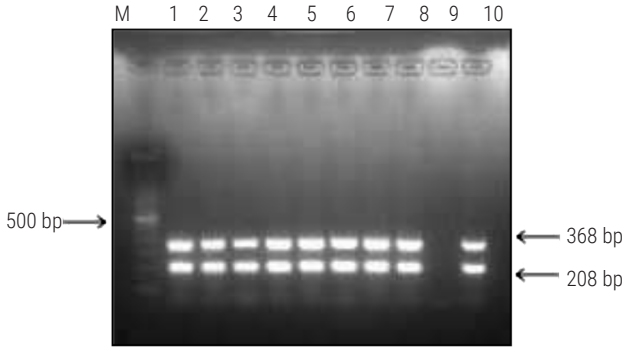

Fig. 1. V_p – *toxR* and *tlh* genes – Agarose gel showing the multiplex PCR product specific to *toxR* and *tlh* genes
Lane M – 100 bp Marker; Lane 1 to 8 – Positive templates; Lane 9 – Negative control; Lane 10 – Positive control

the level of similarity of the toxR between *Vp* and *V. cholera* was only 52%, that was much lesser than that of the rRNA (91 to 92% identity) (Kita-Tsukamoto *et al.,* 1993; Kim *et al.,* 1999). The *toxR* gene was well conserved among *Vp* and therefore, a *toxR*-targeted PCR protocol was customised for the specific detection of *Vp* (Kim *et al.,* 1999). Yang *et al.* (2014) reported that the *Vp* strains causing AHPND were invariably having the transcriptional activator *toxR*. Though the present study revealed the toxR positive *Vp* strains, they were persistently negative to AHPND causing AP1, AP2, AP3 (pirAvp) and AP4 (*pirAvp* and *pirBvp*) genes (Kumar *et al.,* 2014; Ananda Raja *et al.,* 2017b; Das *et al.,* 2017).

Hossain *et al.* (2013) established a multiplex PCR for discovery of *Vp* based on groEL, *tdh* and trh genes with the sensitivity of 200 pg DNA. Similar level of sensitivity was detected in the present study in distinguishing the species-specific marker *tlh* and virulence marker *toxR* genes. The current study discovered that there was not even a single isolate found positive for anthropozoonetic *tdh* and *trh* genes among all the 74 isolates. Sahilah *et al.* (2014) characterised 44 genomic DNA of VP for the occurrence of *toxR*, *tdh* and *trh* genes and found 37 isolates affirmative towards *toxR* gene. No isolate was detected positive to *tdh* and *trh* genes as found in the present study. In disagreement to the present findings, Letchumanan *et al.* (2015) observed that the *trh* gene was found among 6.5% *toxR*-positive isolates. However, no isolates were reported to be affirmative for *tdh* as observed in the present study. As per the reports from various investigators, possession of specific hemolysin genes (*tdh, trh*, or both) was imperative to cause gastroenteritis in human beings (Bej *et al.,* 1999). Conversely, it was also reported that there was a small portion of clinical strains possessed neither of the pathogenic genes, *tdh* and *trh* (Banerjee *et al.,* 2014). Hence, the diagnosis of gastroenteritis caused by *Vp* appears to be challenging. Though the present study revealed that all the isolates were negative for both *tdh* and *trh* genes, it was not ascertained that they were not pathogenic to human during the present study.

The sequences obtained for *toxR* forward (NCBI GenBank accession no. KT360934) and reverse (KT360936) exhibited 99 and 98% homology to Vp RIMD 2210633 chromosome 1, respectively. There was deletion of T at 29th, replacement of A with T at 85th, G with A at 232nd and A with G at 265th positions in forward sequence while there was deletion of T and A at 4th and 30th position and replacement of C with T at 67th, T with A at 190th, A with T at 259th and 292nd positions in reverse sequence, respectively. Both the forward (KT360935) and reverse (KT360937) sequences of *tlh* gene exhibited 96 and 99% homology to Vp RIMD 2210633 chromosome 2. There was deletion of A at 15th, 121st and 160th, replacement of A with C at 118th, C with T at 144th and A with T at 164th positions in forward sequence while there was replacement of T with G at 11th positions in reverse sequence, respectively. Natural deletion and induced mutation were possible in *Vp* as reported by Tinwongger *et al.* (2014) and Nithya Quintal *et al.* (2009), respectively. It needed further validation to find out the possibilities of deletion and mutation changing the isolate pathogenic to shrimp.

It was observed that all the VP isolates were susceptible to AK, GEN, KM, NM, STM, AMC, CEX, CTX, NA, OFLX, MU, SIX, TMP and COT, but resistant to AMP, AMX, P, CIPX, VAN, ERY, NIT and RIF. Antibiotics such as NX, CAP and TCN showed intermediate result against shrimp pathogenic VP isolate as shown in Table 4. The present study revealed that the VP isolates were resistant to AMP, AMX, P, CIPX, VAN, ERY, NIT and RIF. Tendencia and de la Pena (2001) described that the incidence of resistance to oxytetrtacycline (OTC) was highest (4.3%) followed by furazolidone [FZD] (1.6%), OXA (1%) and CAP (0.66%). Han *et al.* (2015) evaluated the antibiotic resistance pattern in Vp strains associated with AHPND in penaeid shrimp and identified a high level of resistance to TCN (≥5 µg ml^{-1}). Luminous *Vibrio* species from shrimp hatcheries in Java Island, Indonesia were confirmed with multiple antibiotic resistances (MAR) to AMP, TCN, AMX, and STM (Tjahjadi *et al.,* 1994).

In agreement with the present findings, Rosalind George, (2002) found that none of the *Vibrio* isolates were sensitive to ERY, but most of the isolates were sensitive to TMP, TCN and NA. As found in the present study, many researchers reported that the V_p isolated from *P. vannamei* was resistant to β-lactam antibiotics (Sahilah *et al.,* 2014; Sudha *et al.,* 2014). Sahilah *et al.* (2014) found that the V_p **isol**ates were not resistant to CIPX and VAN but V_p isolated in the present study was resistant to CIPX and VAN. Antibiotics were not used by the shrimp hatchery operators and shrimp farmers in India aligning with strict implementation of guidelines by the Government of India and awareness created by the Indian Council of Agricultural Research (ICAR) institutes, Marine Product Export and Development Agency (MPEDA), Coastal Aquaculture Authority (CAA), State and Central Government officials, National Centre for Sustainable Aquaculture (NaCSA) as well as Universities (Ananda Raja *et al.,* 2012). Lai

Table 4.Minimum inhibitory concentration (MIC) of V_p isolates from shrimp

Group	Antibiotics	MIC	Result
Aminoglycosides	AK	8 µg	S (≤16)
	GEN	0.25µg	S (≤4)
	KM	3 µg	S (≤16)
	NM	60 µg	NA
	STM	10 µg	NA
β-Lactam Antibiotics	AMP	128 µg	R (>32)
	AMX	>240 µg	R (>8)
	AMC	>240 µg	S (≤8/4)
	P	>256 µg	R (>16)
Cephalosporins	CEX	0.001 µg	NA
	CTX	5 µg	S (≤8)
Fluoroquinolones	CIPX	5 µg	R (>4)
	NA	0.5 µg	S (≤16)
	NX	5 µg	I (5-16)
	OFLX	0.15 µg	S (≤2)
Glycopeptides	VAN	>240 µg	R (>32)
Macrolides	CAP	10 µg	I (9-32)
	ERY	>240 µg	R (>8)
Monoxycarbolic acid	MU	15 µg	NA
Nitrofurans	NIT	240 µg	R (>120)
Rifamycin group	RIF	30 µg	R (>4)
Sulphanomides	SIX	1 µg	NA
	TMP	5 µg	S (≤8)
	COT	4 µg	S (≤2/38)
Tetracyclines	TCN	5 µg	I (5-16)

*Amikacin (AK); Gentamicin (GEN); Kanamycin (KM); Neomycin (NM); Streptomycin (STM); Ampicillin (AMP); Amoxycillin (AMX); Amoxyclav (AMC); Benzyl penicillin (P); Cephalexin (CEX); Cephotaxime (CTX); Ciprofloxacin (CIPX); Nalidixic acid (NA); Norfloxacin (NX); Ofloxacin (OFLX); Vancomycin (VAN); Chloramphenicol (CAP); Erythromycin (ERY); Mupirocin (MU); Nitrofurantoin (NIT); Rifampicin (RIF); Sulphafurazole/Sulfisoxazole (SIX); Trimethoprim (TMP); Co-Trimoxazole (COT); Tetracycline (TCN); S-Susceptible; I-Intermediate; R-Resistant; NA-Not available, so considered as susceptible at that concentration. Reference values are shown in parenthesis.

et al. (2015) reported that antibiotics such as CAP and OFLX were universally effective against both virulent and non-virulent V_p strains, but resistance to AMP in agreement with the present findings. Teo *et al.* (2002) described that the natural occurrence of β-lactamases in *V. harveyi* **s**trains might have caused resistance against β-lactam antibiotics. The same enzyme might also be available in present V_p strain since it belongs to the same genus. Letchumanan *et al.* (2015) reported high resistance of V_p isolates to AMP, AK, KM and CTX but the V_p isolate in the present study was susceptible to AK, KM and CTX but resistant to AMP. In contrast to the present findings, Ananda Raja *et al.* (2017a) reported that there was no unusual antibiotic resistance in *V. mimicus* isolated from diseased shrimp in Sunderban. The present study concluded that the prevalence of V_p in the *P. vannamei* farms was predominant but there was no AHPND causing V_p isolates among the samples collected. Continuous monitoring and screening are required to monitor AHPND in India in future. And also, the multiplex PCR developed targeting *Vp* specific *toxR* and *tlh* genes would be a very much useful to easily identify the shrimp pathogenic non-AHPND *Vp* strains from shrimp farms.

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