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PCR methods characterize AHPND V.p isolates

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Progress in the development of management monitoring strategies for major shrimp disease



Acute hepatopancreatic necrosis disease (AHPND), also been referred to as early mortality syndrome (EMS), has seriously affected farmed shrimp in several countries. Results of this study will aid in the development of management monitoring strategies to limit the spread of the disease and reduce its impact on commercial shrimp farms.

This research analyzed the plasmid sequence from the whole genome sequences of AHPND *V. parahaemolyticus* (*Vp*) – a serious shrimp disease – isolates and identified a clear geographical variation within the plasmid, and developed PCR methods to characterize AHPND *Vp* isolates as either Mexico-type or SE Asia-type. The finding of a geographic sequence variation in the AHPND virulence plasmid can be used as a marker for monitoring the spread of this disease.

In this study, we analyzed the sequence variations in the large virulence plasmid carrying the *pirA*- and *pirB*-like toxin genes, among *Vibrio parahaemolyticus* isolates causing acute hepatopancreatic necrosis disease (AHPND). We found a variable region (4,243-bp) that corresponds to the geographic collection sites of the AHPND isolates. We then developed and applied a duplex PCR assay that can serve to diagnose AHPND and, further, to distinguish among pathogenic AHPND strains collected from various geographic regions.

Sequence variation of the AHPND virulence plasmid

We compared the sequence variation within the virulence plasmid (pVPA3-1) among nine geographic strains (whole genome sequence GenBank no. listed in Table 1). These virulence plasmid's sequences were identified and assembled from the whole genome sequences (WGS) of AHPND-*V. parahaemolyticus* by blast analyses. In Mexico AHPND-*V. parahaemolyticus* strains (M0605 and FIM-

S1708+), the Tn3-like transposon is a 4243-bp fragment inserted in the pVPA3-1's ORF4. However, this fragment is not found in 7 AHPND strains collected from SE Asia including Vietnam, China and Thailand (Table 1, Figure 1).

Isolates	GenBank No.	Origin (year)	Tn3-like transposon
13-028/A3	JOKE000000000	Vietnam (2013)	- (WGSa & PCRb)
NCKU_CV_CHN	JPKU000000000	China (2010)	- (WGS)
NCKU_TV_3HP	JPKS000000000	Thailand (2013)	- (WGS)
NCKU_TV_5HP	JPKT000000000	Thailand (2013)	- (WGS)
TUMSAT_DE1_S1	BAVF000000000	Thailand	- (WGS)
TUMSAT_DE1_S2	BAVG000000000	Thailand	- (WGS)
TUMSAT_D06_S3	BAVH000000000	Thailand	- (WGS)
M0606	JALL000000000	Mexico (2013)	+ (WGS)
FIM-S1708+ (1 isolate*)	JPLV000000000	Mexico (2014)	+ (WGS & PCR)
13-306/D4 (1 isolate*)	NA	Mexico (2013)	+ (PCR)

Tn3-like transposon in Mexico AHPND isolates

To determine if the Tn3-like transposon is only found in the AHPND-*V. parahaemolyticus* isolates collected in Mexico, we selected a pair of PCR primers MX-345F (5'-TACCAGCTCTAACAAGGCCA) and MX-345R (5'-AACGTTCCAAGGAGTCGAGT) to amplify DNA from AHPND isolates in our collections (Table 1). The forward primer is located upstream of the Tn3-like transposon and reverse primer is located within the Tn3-like transposon (Figure 1). Amplifications were performed with the following parameters: initiation denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 7 min. We applied this PCR in 29 pure cultures of AHPND-*V. parahaemolyticus* isolates, and the results showed that all 12 Mexico isolates were positive for the presence of Tn3-like transposon, and none of the 13 Vietnam AHPND isolates were positive for Tn3-transposon (Table 1).

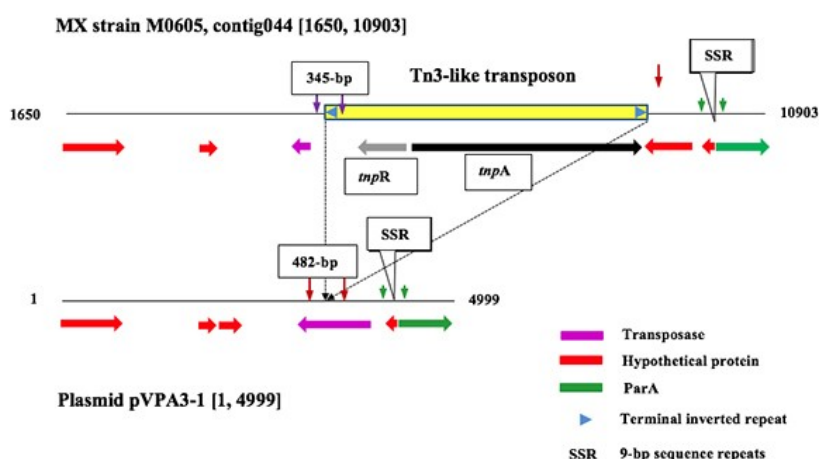


Figure 1. Schematic representation of a Tn3-like transposon in acute hepatopancreatic necrosis disease (AHPND) virulence plasmid. Numbers at the ends indicate nucleotide positions in the contig044 of the Mexico strain M0605 and the plasmid pVPA3-1 harbored in the Vietnam strain 13-028/A3.

Asian AHPND typing PCR

Another pair of PCR primers Asia-482F (5'-TGAACCGTTCCTCATGCTCT) and Asia-482R (5'-TCAAAGCAGCCCAGACAAAC) selected outside the Tn3-like transposon, within the ORF4 of pVPA3-1, was applied to detect AHPND isolates from Vietnam; the amplicon size is expected to be 482-bp for isolates from SE Asia (Figure 1). The results showed that all 13 pure cultures of AHPND isolates from Vietnam were positive and none of the 13 Mexico isolates were positive. These primers can also anneal to Mexico isolates, but the size of amplicons will be 4732-bp, the PCR products were not detected with the PCR cycling profile used.

Duplex PCR for diagnosing and typing AHPND

For simultaneously detecting AHPND and plasmid typing, 2 pairs of primers — MX-345F/R (or Asia-382F/R) and VpPirA-284F (5'-TGACTATTCTCACGATTGGACTG) / VpPirA-284R (5'-CACGACTAGCGCCATTGTTA) — were added into a single tube during PCR. Amplifications were performed with the cycling profile described above. All of the bacterial cultures showed bands at 284-bp indicating the presence of the toxin gene, as expected. Bacterial cultures isolated from Mexico also showed a band at 345-bp, which indicates the presence of the Tn3-like transposon. In contrast, Vietnam AHPND isolates showed a band at 482-bp, which indicates the absence of the Tn3-like transposon (Figure 2).

Figure 2. Duplex PCR detection and typing of acute hepatopancreatic necrosis disease (AHPND) pathogenic isolates of *V. parahaemolyticus* in samples collected in Mexico and Vietnam during 2012 to 2015. Pure bacterial culture isolated from infected shrimp in Mexico (A) and Vietnam (B); DNA extracted from hepatopancreas tissue of infected shrimp collected in Latin America (C) and Vietnam (D).

To test this, we applied the duplex method to 15 DNA samples extracted from hepatopancreas tissues of AHPND-affected shrimp. Eleven shrimp were collected from farms in Latin America countries and four were collected from farms in Vietnam. For the Latin American samples, the duplex PCR showed bands at 345-bp and 284-bp (Figure 2), indicating these shrimp were infected with AHPND

pathogenic bacteria, as shown by the 284-bp band, and that these bacteria contained a Tn3-like transposon. For the 4 samples collected from Vietnam, the duplex PCR resulted in bands of 482-bp and 284-bp (Figure 2), indicating infection with AHPND bacteria that do not have the Tn3-like transposon. Thus, the duplex method described here is useful for rapid diagnosis and typing of AHPND bacteria of infected shrimp collected from ponds.

Virulence comparison between Mexico and Vietnam isolates

Even though it is found on the virulence plasmid of *V. parahaemolyticus*, the T3-like transposon appears to be unrelated to the degree of virulence. We have infected *P. vannamei* with the both Mexico (13-511/A1) and Vietnam (13-028/A3) isolates in the laboratory bioassays. Aquariums (3-L) were filled with artificial seawater at a salinity of 25 ppt and water temperature was maintained at 28°C. Then specific-pathogen free (SPF) *P. vannamei* (mean weight: 1g) were stocked in the aquariums and fed with *V. parahaemolyticus* 13-028/A3 and 13-511/A1, respectively, mixed with shrimp feed. The bacteria were grown to 1×10^9 CFU/mL and mixed with shrimp feed at a 1:1 ratio. The 100 percent cumulative mortality was seen in both isolates by day 3 (Figure 3).

Figure 3. Virulence comparison. Shrimp (*P. vannamei*) were infected Mexico AHPND isolate (13-511/A1) and Vietnam AHPND isolate (13-028/A3) in the laboratory bioassays. All the shrimp became moribund or dead within 3 days and there was no significant difference on AHPND virulence between Mexico and Asia isolates.

Although the function of the genetic variation among geographic isolates of *V. parahaemolyticus* remains unknown, the sequence variation such as the presence or absence a transposon can serve as useful markers for detecting the origins of new outbreaks. There are major variation is the presence of a Tn3-like transposon in the Mexico and Latin America isolates that is absent in Asian isolates. It suggests that the Mexico and Latin America isolates share a common origin. In addition, this will aid in the development of management monitoring strategies to limit the spread of the disease and reduce its impact on commercial shrimp farms.

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