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Genotyping of virulence plasmid from *Vibrio parahaemolyticus* isolates causing acute hepatopancreatic necrosis disease in shrimp

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ABSTRACT: Acute hepatopancreatic necrosis disease (AHPND) has caused severe mortalities in farmed penaeid shrimp throughout SE Asia and Mexico. The causative agent of AHPND is the marine bacterium *Vibrio parahaemolyticus,* which secretes PirA- and PirB-like binary toxin that caused deterioration in the hepatopancreas of infected shrimp. The genes responsible for the production of this toxin are located in a large plasmid residing within the bacterial cells. We analyzed the plasmid sequence from the whole genome sequences of AHPND-*V. parahaemolyticus* isolates and identified 2 regions that exhibit a clear geographical variation: a 4243-bp Tn3-like transposon and a 9-bp small sequence repeat (SSR). The Tn3-like transposon was only found in the isolates from Mexico and 2 unspecified Central American countries, but not in SE Asian isolates from China, Vietnam, and Thailand. We developed PCR methods to characterize AHPND-*V. parahaemolyticus* isolates as either Mexican-type or SE Asian-type based on the presence of the Tn3-like transposon. The SSR is found within the coding region of a hypothetical protein and has either 4, 5, or 6 repeat units. SSRs with 4 repeat units were found in isolates from Vietnam, China, and Thailand. SSRs with 5 repeat units were found in some Vietnamese isolates, and SSRs with 6 repeat units were only found in the Mexican isolates.

KEY WORDS: Tn3-like transposon · Small sequence repeat · Genotying PCR · Shrimp disease · AHPND · *Penaeus vannamei* · Early Mortality Syndrome (EMS)

INTRODUCTION

The adverse effects of a bacterial disease of marine shrimp, acute hepatopancreatic necrosis disease (AHPND, also known as early mortality syndrome, EMS), have caused significant economic losses in shrimp production since the disease was discovered in China in 2009 (Zhang et al. 2012). The causative agents were determined to be unique strains of the bacterium *Vibrio parahaemolyticus* (Tran et al. 2013). This disease then emerged in the SE Asian countries of Thailand, Vietnam, and Malaysia in 2011 (NACA 2012), and was reported in Mexico in 2013 (Soto-Rodriguez et al. 2015).

Molecular studies of this bacterium have shown that the genomes of pathogenic isolates of *V. parahaemolyticus* differ from those of non-pathogenic isolates both in serotype and in DNA pattern (Kongrueng et al. 2014). Subsequent studies showed that the genomes of pathogenic isolates contain 2 genes that are homologous with the *Photorhabdus* insect-related (Pir) toxin genes *pir*A and *pir*B, and which encode PirA&B-like toxin (Yang et al. 2014). These toxin genes were located in a 3.5 kb fragment of a large (69 kb) plasmid that is contained within the *V. parahaemolyticus* genome (Han et al. 2015a). Bioassays with knockout mutants of *pir*A- and *pir*B-like genes have shown that this PirA&B-

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like toxin is the etiological factor for AHPND (G.C.F. Lo pers. comm.).

In this study, based on published whole genome sequences of pathogenic *V. parahaemolyticus* strains, we analyzed the sequence variations within the virulence plasmid. We found 2 variable regions that correspond to the geographic collection sites of the isolates. We then developed and applied a duplex PCR assay that can serve to diagnose AHPND and, further, to distinguish among pathogenic strains collected from various geographic regions.

MATERIALS AND METHODS

AHPND *Vibrio parahaemolyticus* isolates and DNA isolation

AHPND-*V. parahaemolyticus* isolates analyzed and used in this study are shown in Table 1. These include: (1) 9 whole genome sequences (WGSs) available in GenBank. These strains were collected from China, Vietnam, Thailand, and Mexico; (2) 26 bacterial isolates originally from Mexico and Vietnam. These bacterial isolates were sampled from pond water, sediments, and stomachs of shrimp affected by AHPND. Pure cultures were obtained by streaking on tryptic soy agar plus 2% NaCl (TSA+) plates and proved to be pathogenic by laboratory infection as described by Tran et al. (2013). Bacterial identifications were carried out using API Rapid NE test (bioMerieux Industry), 16S rRNA sequencing (Weisburg et al. 1991), and PCR targeting species (*V. parahaemolyticus*)-specific genes (toxR genes) (Kim et al. 1999). These bacteria were stored at −80°C in tryptic soy broth plus 2% NaCl (TSB+) supplemented with sterile glycerol (20% vol/vol); (3) 15 *Penaeus vannamei* samples (each sample consisted of 1 to 5 shrimp) from Vietnam and 2 unspecified Central American countries; these shrimp were collected from AHPND-affected shrimp farms, preserved in 95% ethanol, and sent to our laboratory at the University of Arizona.

For DNA extracted from the pure bacterial culture, each bacterial isolate was grown on TSB+ at 28–29°C with gentle (100 rpm) shaking. The culture was then centrifuged at 5000 × *g* (5 min) and the pellet was resuspended in 1 ml of water, boiled for 10 min. For shrimp preserved in ethanol, the hepatopancreas was sampled and DNA extraction was performed using a QIAamp Tissue Kit (Qiagen) or a Maxwell-16® Cell LEV DNA purification kit (Promega).

Table 1. Isolates of *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease (AHPND) analyzed in this study, indicating detection/non-detection of a Tn3-like transposon and the number of repeat units (RUs) of small sequence repeats (SSRs) in the virulence plasmid pVPA3-1. +: positive; −: not detected; WGS: analyzed from the whole genome sequence; PCR: results from PCR assays; nd: not done

Isolate	GenBank no.	Origin (year)	Tn3-like transposon	RUs of SSR
13-028/A3	JOKE00000000	Vietnam (2013)	– (WGS & PCR[a])	5
NCKU_CV_CHN	JPKU00000000	China (2010)	– (WGS)	4
NCKU_TV_3HP	JPKS00000000	Thailand (2013)	– (WGS)	4
NCKU_TV_5HP	JPKT00000000	Thailand (2013)	– (WGS)	4
TUMSAT_DE1_ S1	BAVF00000000	Thailand	– (WGS)	4
TUMSAT_DE1_ S2	BAVG00000000	Thailand	– (WGS)	4
TUMSAT_ D06_ S3	BAVH00000000	Thailand	– (WGS)	4
M0605	JALL00000000	Mexico (2013)	+ (WGS)	6
FIM-S1708+ (1 isolate[b])	JPLV00000000	Mexico (2014)	+ (WGS & PCR)	6
13-306/D4 (1 isolate[b])		Mexico (2013)	+ (PCR)	6
13-511/A1 (1 isolate[b])		Mexico (2013)	+ (PCR)	6
14-231 (3 isolates[b])		Mexico (2014)	+ (PCR)	6
14-450 (7 isolates[b])		Mexico (2014)	+ (PCR)	nd
12-194G (1 isolate[b])		Vietnam (2012)	– (PCR)	5
14-188 (5 isolates[b])		Vietnam (2014)	– (PCR[a])	4
14-090 (7 isolates[b])		Vietnam (2013)	– (PCR)	nd
13-313 (2 samples[c])		Central America 1 (2013)	+ (PCR)	nd
14-433 (3 samples[c])		Central America 2 (2014)	+ (PCR)	nd
15-133 (6 samples[c])		Central America 2 (2015)	+ (PCR)	nd
12-296 (4 samples[c])		Vietnam (2012)	– (PCR)	nd

[a]PCR using primers MX-345F/R; [b]Pure bacterial culture; [c]Hepatopancreas tissue

Virulence plasmid sequence analysis in AHPND-pathogenic strains

The sequence of a 69 kb virulence plasmid (pVPA3-1, GenBank no. KM067908) was assembled and compared among the WGSs of AHPND-*V. parahaemolyticus* strains using the BLAST program on the NCBI website.

Plasmid typing PCR

PCR was carried out with primers targeting the variable regions of pVPA3-1 (Fig. 1). For the PCR assays, PuReTaq ready-to-go PCR beads were used. The primers targeting a Tn3-like transposon region are: MX-345F (5′-TAC CAG CTC TAA CAA GGC CA) and MX-345R (5′-AAC GTT CCA AGG AGT CGA GT); Asia-482F (5′-TGA ACC GTT CCT CAT GCT CT) and Asia-482R (5′-TCA AAG CAG CCC AGA CAA AC). Amplifications were performed with the following parameters: initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 7 min. Following PCR, an aliquot of PCR products was analyzed in a 2% gel containing ethidium bromide. Amplicons were also sequenced at the University of Arizona sequencing facility; their nucleotide sequences are: (1) KP903899 for a Mexican isolate (13-306/D4) amplified with primers MX-345F/R; (2) KP903893 for a Vietnamese strain (13-028/A3) amplified with primers Asia-482F/R.

Duplex PCR for AHPND detection and plasmid typing

For simultaneous detection of AHPND and plasmid typing, 2 pairs of primers, MX-345F/R (or Asia-382F/R) and VpPirA-284F/R (Han et al. 2015a), were added into a single tube during PCR. Amplifications were performed with the cycling profile described above.

PCR of small sequence repeats

To amplify the region containing small sequence repeats (SSRs), PCR assays were performed using primers SSR-F/R (forward: 5′-CTT TTG CTG TCT CTG GCA CA; reverse: 5′-CCT TTA AAG CTC CCC CAA TC) with the cycling profile described above. Amplified PCR products were sent for DNA sequencing. GenBank accession numbers for SSRs are: (1) KP903895 for the Mexican isolate 13-306/D4; (2) KP903894 for the Mexican isolate 13-511/A1; (3) KP903897 for the Vietnamese isolate 12-194G; (4) KP903898 for the Vietnamese isolate 14-188/1; and (5) KP903896 for the Vietnamese strain 13-028/A3.

The number of repeat units was determined using the Tandem Repeat Finder program (Benson 1999). Alignment of the nucleotide sequences was generated with Clustal X (Thompson et al. 1997).

RESULTS

Virulence plasmid sequence analysis

We compared the sequence variation within the virulence plasmid (pVPA3-1) among 9 geographic strains (WGS GenBank nos. listed in Table 1). These virulence plasmid sequences were identified and assembled from the WGSs of AHPND-*Vibrio parahaemolyticus* by BLAST analyses. We found that the number of SSRs in the plasmid sequences varied with collection site: 4 repeat units (RUs) in strains from China and Thailand, 5 RUs in a Vietnamese strain, and 6 RUs from Mexican strains. In addition, another distinct sequence variation, the presence of a Tn3-like

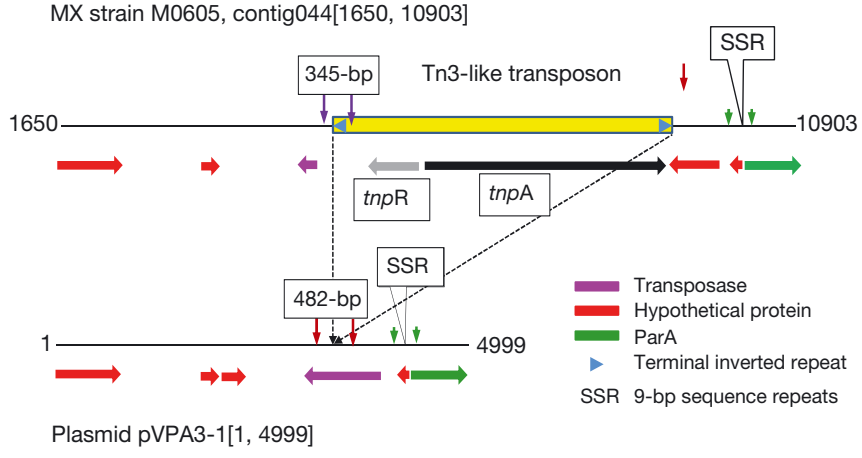

Fig. 1. Representation of a Tn3-like transposon and small sequence repeats (SSRs) in acute hepatopancreatic necrosis disease (AHPND) virulence plasmids. Numbers at the ends indicate nucleotide positions in the contig044 of the Mexican *Vibrio parahaemolyticus* strain M0605 and the plasmid pVPA3-1 harbored in the Vietnamese strain 13-028/A3

transposon, was found only in the 2 Mexican strains (M0605 and FIM-S1708+).

During the analysis, we also found a discrepancy in the WGS of 1 AHPND-*V. parahaemolyticus* strain from Mexico in GenBank. This strain, FIM-S1708+, did not contain the expected *pir*A&B-like genes. The published WGS of this strain only contains 90% of the *pir*B-like gene, and the sequence for the *pir*A-like gene was not found. However, we later concluded that the missing *pir*A&B-like gene segments were most likely due to errors in sequencing or assembly. We obtained this strain and were able to detect both *pir*A&B-like genes with the PCR method described by Han et al. (2015a). The poor quality of the WGS of FIM-S1708+ is also evidenced by comparison with another Mexican strain, M0605. In the WGS of FIM-S1708+, 2/3 of the pVPA3-1 sequence is missing; we only found a 26 kb nucleotide sequence that showed 99.9% identity to the plasmid. In contrast, the WGS of M0605 contains 3 large contigs (21–42 kb) and reveals a 69 kb sequence with 99.0 to 99.9% identities to pVPA3-1.

Tn3-like transposon

In Mexican AHPND-*V. parahaemolyticus* strains, the Tn3-like transposon is a 4243 bp fragment inserted in the open reading frame 4 (ORF4) of pVPA3-1. This fragment is not found in 7 AHPND strains collected from SE Asia (Table 1). From the WGS of Mexican strains, this 4.2 kb fragment is located in contig044 (GenBank no. JALL01000058, 21.1 kb) of strain M0605, and in contig51 (JPLV01000051, 10.7 kb) of strain FIM-S1708+. This transposon contains a 38 bp inverted terminal repeat (ITR; GGG GTT TGA GGT CCA ACG GAA CGA AAG TGT ACG TTT AG), this ITR is rather conserved among bacterial plasmids, as the sequence has 89 to 91% identities to plasmids residing within several bacteria, including *Aeromonas salmonicida*, *Escherichia coli*, *Enterobacter cloacae*, and others.

By BLAST analysis, this 4.2 kb transposon was found to be a member of the Tn3 superfamily. Its insertion disrupts ORF4 of pVPA3-1; ORF4 is a putative transposase gene (GenBank no. AIL49931, 314 aa) that belongs to the transposase_31 superfamily. In strain M0605, the disrupted transposase gene (*tnp*) split into 2 ORFs encoding a small transposase (ETZ12328, 88 aa) and a hypothetical protein (ETZ12335, 152 aa; Fig. 1).

This Tn3-like transposon contains 2 genes, *tnp*A and *tnp*R, encoding a transposase (1000 aa, ETZ12331) and a serine-based recombinase (185 aa, WP_031419876), respectively. The TnpA transposase has a DDE_Tnp_Tn3 domain (390 aa) at the C-terminus, and it has 61 to 77% identities to transposases in the plasmids of *E. coli*, *Klebsiella pneumonia*, *Yersinia pestis*, and others. The TnpR serine-based recombinase contains a 125 aa SR_ResInv domain at the N-terminus, and a DNA-binding domain at the C-terminus, and it has ~77% identities to those found in plasmids of various bacteria including *Yersinia* spp., *Klebsiella* spp., *Salmonella* spp., and *E. coli*, among others. In this study, we analyzed the *tnp*R gene based on the sequence of contig51 from strain FIM-S1708+. In strain M0605, this gene was split into 2 ORFs (ETZ12329, 133 aa; ETZ12330, 52 aa) resulting from 1 nucleotide deletion which is most likely a sequencing error. The homologues of this recombinase from other bacteria consist of 182 to 187 aa.

PCR detection of Tn3-like transposon in AHPND-*V. parahaemolyticus* isolates

To determine whether 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/R) to amplify DNA from AHPND isolates in our collections. The forward primer is located upstream of the Tn3-like transposon, and the reverse primer is located within the Tn3-like transposon (Fig. 1). The amplicon size was expected to be 345 bp. We applied this PCR in 26 pure cultures of AHPND-*V. parahaemolyticus* isolates, and the results showed that all 13 Mexican isolates were positive for the presence of the Tn3-like transposon, whereas none of the 13 Vietnamese AHPND isolates was positive for the Tn3-transposon (Table 1).

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

Duplex PCR for diagnosing and typing AHPND-*V. parahaemolyticus*

For diagnosing and typing AHPND isolates, we combined the typing PCR that detects the presence or absence of the Tn3-like transposon with a diagnostic PCR assay that detects the *pir*A-like toxin gene to form a duplex PCR method. We applied this method to 26 pure AHPND-*V. parahaemolyticus* isolates from Mexico and Vietnam. 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 (Fig. 2A). In contrast, Vietnamese AHPND isolates showed a band at 482 bp, which indicates the absence of the Tn3-like transposon (Fig. 2B). These results, with pure bacterial cultures, will be most useful if the duplex PCR method can be successfully applied to routine DNA samples extracted from shrimp tissues, as commonly received in diagnostic laboratories.

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 2 Central American countries, and 4 were collected from farms in Vietnam. For the Central American samples, the duplex PCR showed bands at 345 and 284 bp (Fig. 2C), indicating that 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 and 284 bp (Fig. 2D), 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.

With this duplex PCR analysis of a non-pathogenic strain (13-028/A2), neither the *pir*A-like gene nor the Tn3-like transposon was detected. This strain does not contain the virulence plasmid pVPA3-1 (Han et al. 2015a).

Variable numbers of SSRs

An SSR region within the virulence plasmid, identified at nucleotides 4240–4284 within the coding region of ORF5, contains a 9 bp sequence repeat, 'TTG TTT TTC' (Fig. 3). This SSR was not found in the other bacterial genomic sequences. From the WGS, there are 4 RUs for AHPND strains from Thailand and China, 5 RUs from a Vietnamese strain, and 6 RUs from Mexican strains (Fig. 3, Table 1). We applied a PCR to amplify this region from pure cultures of AHPND-*V. parahaemolyticus* isolates collected from Vietnam and Mexico, and the size of the amplicons was approximately 580 bp. By DNA sequencing, these amplified fragments were found to contain 4, 5, and 6 RUs (Fig. 3, Table 1). One Vietnamese isolate (12-194G) contained 5 RUs. Five isolates (14-188/1–5) from Vietnam contained 4 RUs. The 6 RUs were only found in 5–6 isolates (FIMS1708+), (13-306/D4, 13-511/A1, 14-231/B23, 14-231/B25, and 14-231/D74) from Mexico.

DISCUSSION

Geographic sequence variations in the virulence plasmid (pVPA3-1) found among AHPND-*Vibrio parahaemolyticus* isolates can be used as markers for

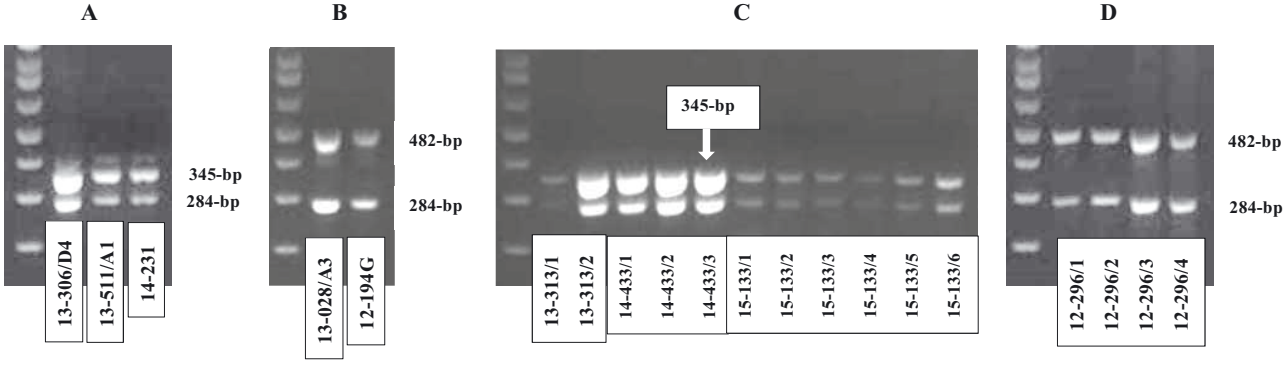

Fig. 2. Duplex PCR detection and typing of acute hepatopancreatic necrosis disease (AHPND) pathogenic isolates of *Vibrio parahaemolyticus* in samples collected in Mexico and Vietnam during 2012 to 2015. Pure bacterial culture isolated from infected shrimp in (A) Mexico and (B) Vietnam; DNA extracted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

Fig. 3. Multiple alignment of nucleotide sequences corresponding to small sequence repeat (SSR, TTGTTTTTC) regions from acute hepatopancreatic necrosis disease (AHPND) pathogenic isolates of *Vibrio parahaemolyticus*. Repeat units are marked within boxes in each isolate, dashes indicate gaps

monitoring the spread of this disease. There are minor differences in SSR number between geographic isolates, but the major variation is the presence of a Tn3-like transposon in the Mexican and Central American isolates that is absent in others. This suggests that the Mexican and Central American isolates share a common origin.

Although useful as a genetic marker, the function of this Tn3-like transposon is not clear. This Tn3-like transposon contains 2 genes (*tnp*A and *tnp*R) and a *cis*-acting site (38 bp inverted terminal repeats). The *tnp*A gene encodes the DDE-transposase, which is required for the cointegration of donor and recipient replicons. The *tnp*R gene encodes a recombinase that breaks down the cointegrate into separate donor and recipient replicons (Heffron et al. 1979, Maekawa & Ohtsubo 1994). The *tnp*A and *tnp*R sequences of the Tn3-like transposon have not been described in other species of *Vibrio*, but they are 70% similar to transposons found in strains of *E. coli.*

Even though it is found on the virulence plasmid of *V. parahaemolyticus,* the transposon appears to be unrelated to AHPND. We infected *Penaeus vannamei* with Mexican (13-306/D4) and Vietnamese (13-028/A3) isolates in the laboratory bioassays, and 100% cumulative mortality was seen in both isolates by Day 3 (Han et al. 2015b). Also, in other studies, we identified plasmids containing this Tn3-like transposon in 2 non-pathogenic *V. parahaemolyticus* isolates from Mexico (authors' unpubl. data). These 2 non-pathogenic isolates tested positive through PCR typing, showing the presence of the Tn3-like transposon, but *pir*A&B-like toxin genes were not detected. These non-pathogenic isolates can be considered natural *pir*A&B(-) strains and were not infectious in laboratory bioassays (authors' unpubl. data). Transposons can jump randomly between genomes or plasmids, resulting in gene transfers (Kleckner 1981, Muñoz-López & Garcia-Perez 2010, Zhang et al. 2011), and usually carry genes that are beneficial to their hosts, such as for toxin production or antibiotic resistance; however, the Tn3-like transposon of Mexican isolates only contained 2 transposition genes. Although the function, if any, of the genetic variation among geographic isolates of *V. parahaemolyticus* remains unknown, these variations, such as the presence or absence a transposon and differences in SSR number, allow the characterization and differentiation of pathogenic strains. There may be differences among strains in their interaction with the host, resulting in differences in virulence which may prove valuable in the development of resistant lines of shrimp. In addition, these genetic characteristics of individual strains can serve as markers useful in detecting the origins of new outbreaks. Following the genetic variations of individual strains will aid in understanding the evolution of the pathogen, which is necessary for keeping diagnostic and management strategies up to date and, subsequently, to limit the spread of the disease and reduce its impact on commercial shrimp farms.

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