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First report of acute hepatopancreatic necrosis disease (AHPND) occurring in the USA

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ABSTRACT: In June 2017, mass mortalities were reported at whiteleg shrimp *Penaeus vannamei* farms in Texas, USA. PCR testing for OIE-listed and non-listed pathogens detected the *pirA* and *pirB* toxin genes associated with acute hepatopancreatic necrosis disease (AHPND). DNA sequence analyses of cloned *pirA* and *pirB* genes showed them to be identical to those detected in other AHPND-causing *Vibrio* sp. Amplicons generated using PCR tests targeted to the *toxR* gene showed the Pir toxin genes to be associated with a *V. parahaemolyticus* type more similar to a genotype found in Mexico compared to that found in Asia. Histology detected masses of bacteria and hemocytic infiltrations as well as extensive necrosis and sloughing of epithelial cells in hepatopancreatic tubules pathognomonic of AHPND. The data support AHPND as the cause of the mortalities. Given that US companies produce shrimp broodstock for farms in Asia and Latin America, the further spread of AHPND in the USA needs to be prevented to avoid serious economic consequences to these industries.

KEY WORDS: Acute hepatopancreatic necrosis disease · AHPND · Early mortality syndrome · EMS · *Penaeus vannamei* · Shrimp aquaculture

1. INTRODUCTION

Acute hepatopancreatic necrosis disease (AHPND) is the primary cause of early mortality syndrome (EMS) that has emerged recently as a major threat to shrimp aquaculture worldwide (Lightner et al. 2012, Tran et al. 2013, Flegel 2014, Joshi et al. 2014, Hong et al. 2016). The disease first appeared in China in 2009, and since then has appeared in Vietnam in 2010 (Tran et al. 2013), Malaysia in 2011 (FAO 2013), Thailand in 2012 (Joshi et al. 2014), Mexico in 2013 (Nunan et al. 2014, Soto-Rodriguez et al. 2015), the Philippines in 2014 (de la Peña et al. 2015), and more recently in Bangladesh (Eshik et al. 2017) and in Myanmar (Tun et al. 2017). Since its recognition, AHPND has been estimated to have caused >$20 billion USD in losses to shrimp aquaculture in Asia (www.undercurrentnews.com/2016/09/09/disease-has-cost-asia-shrimp-sector-over-20bn/). In Thailand alone in 2013, a ~30% drop in shrimp production was attributed to AHPND at farms along the western coast of the Gulf of Thailand (www.fisheries.go.th/ems/).

Mortalities caused by AHPND generally occur within the first 5 wk of stocking a pond with post-larvae, although there is a report of it killing late-stage juveniles cultured for ~3 mo (de la Peña et al. 2015). Clinical signs of the disease include lethargy, slow growth, an empty stomach and midgut, and a pale to white atrophied hepatopancreas, with dead shrimp

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often amassing at the pond bottom. The histological hallmark of AHPND is the rounding and sloughing of epithelial cells in hepatopancreatic tubules in the absence of any causative pathogen. In the later stage of disease, hemocytic aggregations and melanized granulomas appear in hepatopancreatic tubules accompanied by opportunistic bacteria invasions (Tran et al. 2013). While AHPND has affected the 2 major species of farmed shrimp, i.e. *Penaeus vannamei* and *P. monodon*, it has also been detected recently in the farmed freshwater prawns *Macrobrachium rosenbergii* (Tun et al. 2017).

AHPND was shown initially to be caused by a specific strain of *Vibrio parahaemolyticus* described as VP_{AHPND} (Zhang et al. 2012, Tran et al. 2013, Soto-Rodriguez et al. 2015). The pathology associated with AHPND was subsequently found to be due a deadly Pir^{VP} toxin expressed from a plasmid (pVA1) carried by VP_{AHPND} strains (Lee et al. 2015). This toxin comprises 2 subunits, PirA and PirB, and is homologous to the *Photorhabdus* insect-related (Pir) binary toxin (Han et al. 2015a, Lee et al. 2015). The pVA1 plasmid also carries a cluster of genes to facilitate conjugative transfer among *V. parahaemolyticus* strains (Lee et al. 2015) or to other *Vibrio* species including *V. owensii* (Liu et al. 2015), *V. campbellii* (Dong et al. 2017), *V. harveyi* (Kondo et al. 2015), and *V. punensis* (Restrepo et al. 2018).

In 2016, total production of farmed shrimp in the Rio Grande Valley, Texas (USA), was 296 t. In 2017, however, production decreased by almost 40% to 114.4 t, with AHPND being hypothesized to be the most likely cause of the losses (R. Adami Jr & Y. S. Juan unpubl.). To confirm the cause, in June 2017, juvenile *P. vannamei* collected at farms in Cameron County, Texas, experiencing mass mortalities and birds feeding on shrimp at the water surface were provided to The University of Arizona for analysis. Shrimp samples were collected by field biologists of the Texas Park and Wildlife Department. Described here are histological, PCR, and DNA sequencing data indicating that the mortality event was caused by an AHPND-causing *V. parahaemolyticus* strain similar to a genotype detected in Mexico but not in Asia. This is the first report confirming the presence of AHPND-causing *V. parahaemolyticus* in the USA.

2. MATERIALS AND METHODS

2.1. Samples

During the summer of 2017, 3 *P. vannamei* farms in Cameron County, Texas, experienced large-scale mortalities. Shrimp were 15–21 g in weight at the time. At Farm 1, mortality occurred at 72 d post-stocking, and at Farms 2 and 3, mortality occurred at 89 d post-stocking. From Farm 1, samples were collected during emergency harvest. Five whole shrimp were preserved in 95% ethanol and 5 additional shrimp were preserved in Davidson's alcohol-formalin-acetic acid for histology.

2.2. Nucleic acid extraction and PCR screening for viral, bacterial, and fungal diseases

Total DNA was extracted from either hepatopancreas (HP) tissue sampled from each shrimp or from a pool of pleopod and HP tissue sampled from all 5 shrimp using a Maxwell 16 cell LEV DNA Purification Kit (Promega) and the Promega cell DNA extraction protocol. Similarly, total RNA was extracted from pooled samples (n = 5) of pleopod tissue using a Maxwell 16 cell LEV RNA Purification Kit (Promega) and the Promega cell RNA extraction protocol.

Pleopod DNA was amplified using PCR tests for infectious hypodermal and hematopoietic necrosis virus (IHHNV; Tang & Lightner 2006), IHHNV-related genome-integrated sequence (Tang et al. 2007), and white spot syndrome virus (WSSV; Durand & Lightner 2002). Hepatopancreas DNA was amplified using PCR tests for *Baculovirus penaei* (BP; D. V. Lightner et al. unpubl.), necrotizing hepatopancreatitis bacterium (NHP-B; Aranguren et al. 2010), *Enterocytozoon hepatopenaei* (EHP; Tang et al. 2015), and AHPND (see Section 2.3). Pleopod RNA was amplified using PCR tests for yellow head virus (YHV; Aranguren et al. 2012), Taura syndrome virus (TSV; Tang et al. 2004), and infectious myonecrosis virus (IMNV; Andrade et al. 2007).

2.3. AHPND PCR tests

pirA and *pirB* toxin gene sequences associated with AHPND were detected by duplex PCR using a *pirA* gene-specific primer pair VpPirA-284F:VpPirA-284R (284 bp amplicon) in combination with a *pirB* gene-specific primer pair PirB-F:PirB-R (392 bp amplicon) as described in the OIE Manual of Diagnostic Tests for Aquatic Animals (Han et al. 2015b, OIE 2017). DNA was amplified in a 25 µl reaction using PuReTaq Ready-to-Go PCR beads (GE Healthcare), 0.4 µM pirA-specific and 0.2 µM pirB-specific PCR primers, and 1 µl (~40 ng) DNA. The thermal cycling conditions used were 94°C for 3 min, 35

cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; followed by 72°C for 5 min.

A nested PCR employing the AP4 primer set was also run as described in the OIE manual (OIE 2017). DNA products amplified by each PCR were electrophoresed in 1.5% agarose gels containing 0.5 µg ml^{-1} ethidium bromide and visualized/recorded using a Bio-Rad Gel-Doc™ XR+ UV Imaging System.

2.4. PCR tests to identify *Vibrio* species and genotype *V. parahaemolyticus*

Vibrio species were identified by PCR using primer pairs targeting *toxR* gene sequences specific to *V. harveyi* (harv-F:harv-R, 967 bp amplicon) (Fukui & Sawabe 2007), *V. parahaemolyticus* (toxR-VP-F: toxR-Vp-R, 368 bp amplicon) (Kim et al. 1999), and *V. campbellii* (Vca-hly5:Vca-hly3, 328 bp amplicon) (Haldar et al. 2010). The PCR test reported by Han et al. (2015b) was used to distinguish Mexican and Asian *V. parahaemolyticus* genotypes.

2.5. Cloning and sequencing of *pirA* and *pirB* genes

Hepatopancreas DNA was amplified by PCR using either the full-length *pirA* gene primers pirAFull-1F (5′-ATG AGT AAC AAT ATA AAA CAT GAA ACT GAC-3′) and pirAFull-1R (5′-TTA GTG GTA ATA GAT TGT ACA GAA ACC-3′) or the full-length *pirB* gene primers pirBFull-1F (5′-ATG ACT AAC GAA TAC GTT GTA ACA ATG-3′) and pirBFull-1R (5′-CTA CTT TTC TGT ACC AAA TTC ATC GG-3′). Reaction mixtures comprised PuReTaq Ready-To-Go PCR Beads, 1.0 µl of 10 µM primer mix and 1.0 µl DNA in a 25 µl reaction volume. Thermal cycling conditions used for the *pirA* PCR were 95°C for 3 min followed by 30 cycles of 95°C for 30 s, 55°C for 20 s, and 72°C for 60 s, followed by 72°C for 5 min. Cycling conditions for the *pirB* PCR were the same except for use of a longer (90 s) final extension step. DNA products were detected in a 1% agarose gel as above, recovered using a Qiagen gel extraction kit and ligated into TOPO pCR2.1. Following transformation of *E. coli* cells, clones containing recombinant plasmid DNA were identified, and plasmid DNA was isolated using a Qiagen DNA miniprep kit. Plasmid DNA was sequenced in both directions. The full-length DNA sequences of *pirA* (MH410659) and *pirB* (MH410660) were deposited in GenBank and translated using an ExPASy translation tool. The MUSCLE program was used to perform multiple alignments of the predicted amino acid sequences of the *pirA* and *pirB* genes determined for the *V. parahaemolyticus* Texas strain together with homologues from AHPND-causing *V. parahaemolyticus* strains from other geographical regions.

2.6. Histology

Fixed tissues from the 5 shrimp were processed, embedded in paraffin, and sectioned (4 µm thick) in accordance with standard histological methods. Tissue sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy. Severity of AHPND histopathology was graded as G0 (absence) to G4 (severe lesions and advanced tissue destruction) as described previously (Lightner 1996).

3. RESULTS

3.1. PCR screening for viral, bacterial, and fungal diseases

PCR testing of either pleopod DNA or pleopod RNA or HP DNA extracted from *Penaeus vannamei* pooled samples (n = 5) collected in June 2017 from Farm 1 in Texas experiencing mass mortalities did not detect IHHNV, WSSV, BP, TSV, IMNV, YHV, the non-infectious genome-integrated IHHNV sequence, NHP, or EHP. In contrast, an AHPND duplex PCR co-amplifying the *pirA* and *pirB* genes generated amplicons consistent with their expected size (284 and 392 bp, respectively) with DNA extracted from the HP tissue pool (Fig. 1a). The AP4 nested PCR method designed to amplify a sequence including the junction of *pirA* and *pirB* genes also generated a DNA amplicon of the expected size (230 bp) with this DNA extract (Fig. 1a). When HP DNA extracted from each of the 5 shrimp was tested separately using the duplex AHPND PCR, DNA amplicons of the size expected for *pirA* and *pirB* were detected in 1 out of 5 shrimp.

3.2. *Vibrio* species identification and genotyping of AHPND-causing *V. parahaemolyticus*

Vibrio species-specific PCR primers were used to confirm the *Vibrio* sp. associated with AHPND. This PCR test amplified a DNA product of a size (368 bp) expected for *V. parahaemolyticus*, but no products of sizes expected for either *V. harveyi* or *V. campbellii* (Fig. 1b).

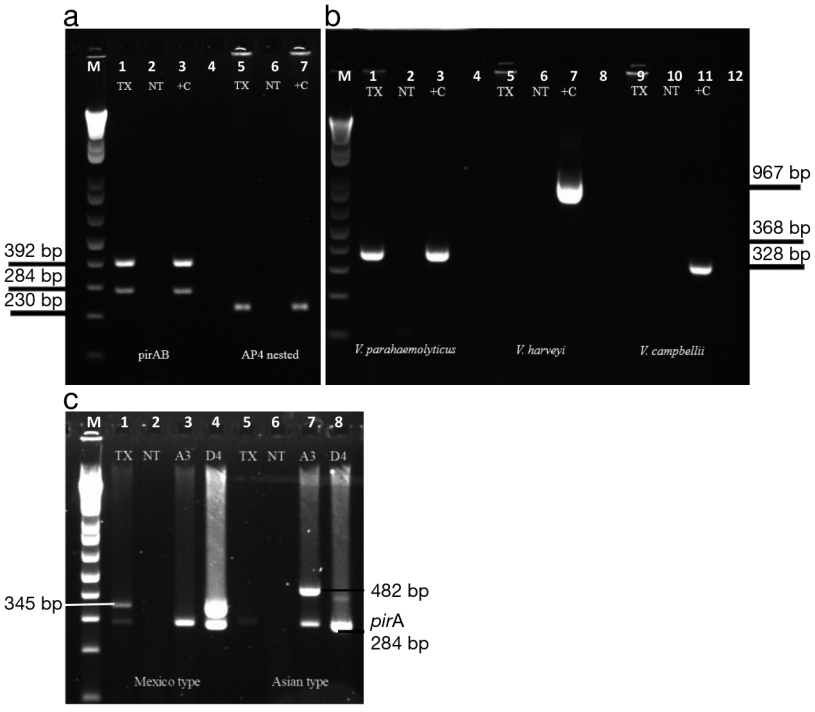

Fig. 1. (a) Duplex PCR co-amplifying *pirA* (284 bp) and *pirB* (392 bp) genes of *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease (AHPND) (Lanes 1–3) and AP4 nested PCR product (230 bp) obtained using *pirA* and *pirB*-specific primers (Lanes 5–7), from hepatopancreas DNA extracted from shrimp in Texas (TX, Lanes 1 and 5), a no-template control (NT, Lanes 2 and 6), and an AHPND-positive shrimp DNA control (+C, Lanes 3 and 7). (b) *Vibrio* species-specific DNA products amplified from hepatopancreas DNA extracted from shrimp in Texas (TX, Lanes 1, 5, 9), NT (Lanes 2, 6, 10), and *V. parahaemolyticus* DNA (+C, Lane 3), *V. harveyi* (+C, Lane 7), and *V. campbellii* (+C, Lane 11) using *toxR* gene primers specific to *V. parahaemolyticus* DNA (Lanes 1–3, 368 bp product), *V. harveyi* (Lanes 5–7, 967 bp product), and *V. campbellii* (Lanes 9–11, 328 bp product). Lanes 4 and 8 are empty. (c) *V. parahaemolyticus* genotype-specific PCR amplicons detected using primers specific for *V. parahaemolyticus* strains from Mexico (Lanes 1–4) or Asia (Lanes 5–8), using hepatopancreas DNA extracted from shrimp in Texas (TX, Lanes 1, 5), and NT (Lanes 2 and 6). DNA product sizes for the strains from Mexico (D4, 345 and 284 bp) and Asia (A3, 482 and 284 bp). M: 1 kb DNA size marker

V. parahaemolyticus genotyping PCR primers amplified a 345 bp DNA product from Texas *P. vannamei* (Case No. 327) and a strain (D4) detected at a farm in Mexico, but not from the A3 Asian strain (Fig. 1c), and a 482 bp DNA product was amplified from this Asian isolate but not from the Mexican or Texas strains (Fig. 1c). A 284 bp DNA was amplified from all geographic isolates (Fig. 1c).

3.3. Sequence analysis of the *pirA* and *pirB* genes

PCR amplification and sequence analysis of the *pirA* and *pirB* open reading frames (ORFs) showed their predicted amino acid sequences to be identical to those detected in AHPND-causing *V. parahaemolyticus* strains from Thailand, Vietnam, Taiwan, and Mexico (data not shown).

3.4. Histopathology

Histopathology observed in HP tissue from the 5 *P. vannamei* are shown in Fig. 2. In 1 shrimp, no lesions characteristic of AHPND were evident, and highly vacuolated lipid droplets were prominent in HP tubule cells (Fig. 2a). In the other 4 shrimp, lesions characteristic of acute terminal-phase AHPND were evident. These included the absence of lipid droplets (Grade G0) and B and R cells in HP tubules (Fig. 2b–d) as well as multifocal necrosis and massive sloughing of tubule epithelial cells in the HP medial region (Fig. 2b,c), sometimes in association with hemocytic inflammations (Fig. 2c) or bacterial infection in the HP tubule lumen and hemocytic infiltrations surrounding affected tubules (Fig. 2d). No histopathology indicative of viral or other known bacterial pathogens was detected.

4. DISCUSSION

Of the pathogens tested for by PCR, only APHND was detected as the etiologic agent involved in mass mortalities that occurred at a *Penaeus vannamei* farm in Texas during the summer of 2017. Both the AHPND duplex PCR designed to co-amplify the *pirA* and *pirB* toxin genes (OIE 2017) and the AP4 PCR designed to amplify a region spanning the *pirA–pirB* gene junction produced DNA products of the expected sizes. While both the *pirA* and *pirB* genes, specified for specific PCR diagnosis of AHPND, were detected using DNA extracted from a pooled HP tissue sample from 5 shrimp, evidence of both genes was only found in 1 of these 5 animals. However, when tested by H&E histology, the other 4 out of 5 animals displayed the terminal phase of AHPND. Thus, combining the samples tested using 2 different detection methods, 5 out of 10 animals were infected with AHPND. It is possible that the infection had not yet reached its peak when the emergency harvest was conducted. We cloned and sequenced

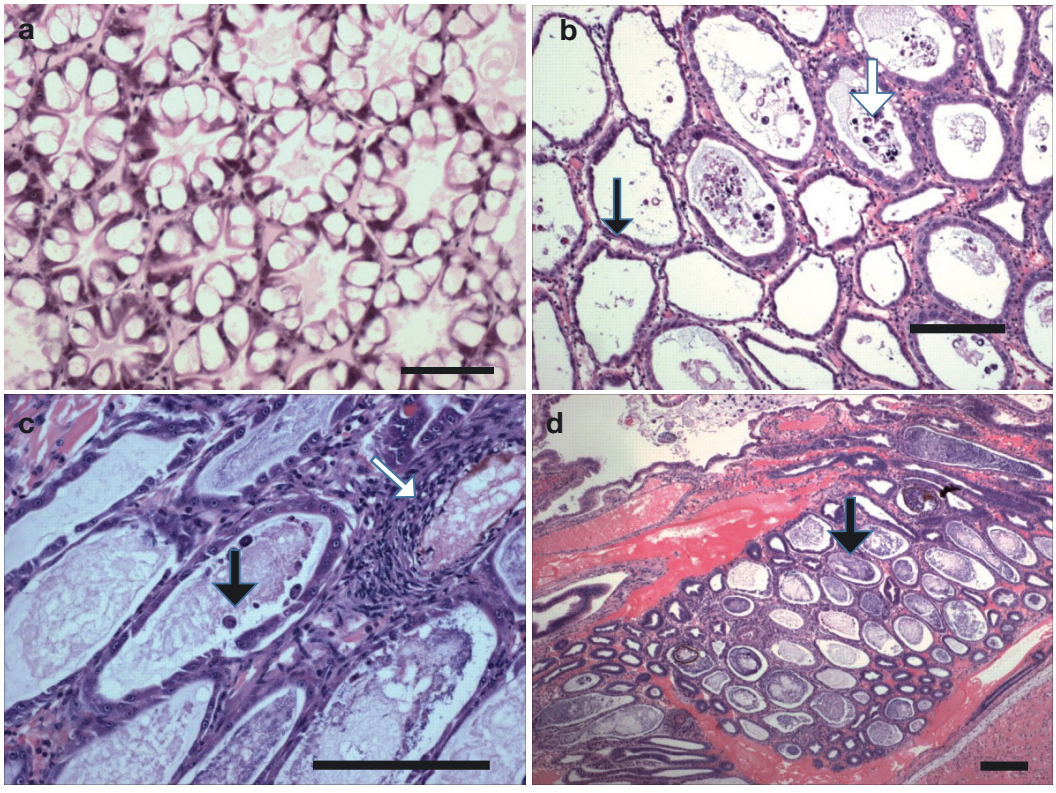

Fig. 2. Histopathology detected in hepatopancreas (HP) tissues of a *Penaeus vannamei* from a Texas farm experiencing mortalities. Tissue sections stained using Mayer-Bennett's H&E. Scale bars = 100 µm. (a) Normal HP tubule epithelium; (b) acute sloughing of HP tubule epithelial cells (white arrow), atrophy of HP tubule (small arrow); (c) sloughing of HP tubule cells (large arrow), hemocytic inflammation (white arrow); (d) acute hepatopancreatic necrosis disease terminal phase characterized by massive bacterial infestation associated with the necrotic and sloughed tubule cells in the HP lumen (arrow)

pirA and *pirB* genes, and the predicted amino acid sequences determined for the *pirA* and *pirB* gene ORFs were identical to those found in AHPND-causing *Vibrio* sp. detected elsewhere.

Histology of cephalothorax tissues from the 5 shrimp examined revealed extensive necrosis and sloughing of epithelial cells in the HP tubule and bacterial accumulations associated with hemocytic infiltrations in the lumen of affected HP tubules. These observations are typical of the terminal phase of AHPND as supported by the molecular data. While known to be caused by different species of *Vibrio* (Tran et al. 2013, Kondo et al. 2015, Liu et al. 2015, Dong et al. 2017, Restrepo et al. 2018), *Vibrio* species-specific PCR tests confirmed *V. parahaemolyticus* to be the AHPND etiologic agent, and to be more similar to the Mexican type than to the Asian type (Han et al. 2015b). In shrimp aquaculture, pathogens are often translocated via movements of broodstock or post-larvae with unapparent infections (Karunasagar & Ababouch 2012). While the data suggest some linkage to AHPND-causing *V. parahaemolyticus* species found in Mexico, its origin and how it arrived in Texan shrimp farms remains to be determined.

AHPND is a major threat to shrimp farming worldwide and since first being detected in China in 2009, its distribution has expanded to many countries in Asia and also to Mexico and as reported here, the USA, in the Western Hemisphere. OIE notification of the detection of AHPND in the USA in 2017 has impacted not only shrimp farms producing commodity shrimp in Texas, but also US companies producing shrimp feed and feed additives (www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapFullEventReport&reportid=24597). Firstly, disease caused by AHPND has reduced the feed needs of farms, and secondly, shrimp feed producers now also need to assimilate additional AHPND testing costs for feed products shipped to Asia and Latin America. Moreover, to prevent its spread to other shrimp farms and broodstock producers in Texas and elsewhere, surveillance for the presence of AHPND in farmed and wild shrimp popula-

tions will be critical in assessing and managing potential risks that these pose. As companies producing shrimp broodstock located in Florida and Hawaii as well as Texas supply genetically selected and specific pathogen free *P. vannamei* broodstock to farms in Asia and Latin America, the further spread AHPND in the USA needs to be prevented to avoid dire economic consequences to these industries.

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