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NOTE

Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico

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ABSTRACT: Acute hepatopancreatic necrosis disease (AHPND), which has also been referred to as early mortality syndrome (EMS), initially emerged as a destructive disease of cultured shrimp species in Asia in 2009. The pathogen associated with the disease, *Vibrio parahaemolyticus*, subsequently spread to the Western Hemisphere and emerged in Mexico in early 2013. The spread to the Western Hemisphere is a major concern to shrimp producers in the region. To date, the only peer-reviewed published method for determining whether mortalities are due to AHPND is through histological examination. A novel PCR detection method was employed to assess samples from Mexico in order to confirm the presence of the pathogen in this country. This manuscript details the detection methods used to confirm the presence of AHPND in Mexico. Both immersion and per os challenge studies were used to expose the *Penaeus vannamei* to the bacteria in order to induce the disease. Histological analysis confirmed AHPND status following the challenge studies. Also provided are the details of the molecular test by PCR that was used for screening candidate *V. parahaemolyticus* isolates. A rapid PCR assay for detection of AHPND may help with early detection and help prevent the spread of AHPND to other countries.

KEY WORDS: AHPND · Early mortality syndrome · EMS · *Vibrio parahaemolyticus* · Shrimp disease

INTRODUCTION

The emergence of a new bacterial pathogen has been dramatically affecting the culture of *Penaeus vannamei* in the Southeast Asian countries of China, Vietnam, Malaysia and Thailand (Leaño & Mohan 2012, NACA 2012, FAO 2013, de Schryver et al. 2014). The pathogen, determined to be a *Vibrio parahaemolyticus* strain by Tran et al. (2013), most recently has decimated *P. vannamei* culture in the northern states of Mexico, including Nayarit, Sinaloa and Sonora. The shrimp producers' associations in those states projected a 65% drop in tons of shrimp produced in 2013 when compared to 2011 (The Fish Site 2013).

During the 2013 culture cycle in NW Mexico, >1500 farms were in operation, most of them performing 2 cycles per year, with a total production area of 95 962 ha. Mortalities first began in Nayarit State, located in the southern area of the affected region (Fig. 1), in March. As the northern regions of Sinaloa and Sonora States began stocking shrimp postlarvae (PL), mortalities began there. In Sinaloa, mortalities began in March, reached a maximum by May, and decreased in the following months; in Sonora, mortalities began in May, reaching a maxi-

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mum by June, and caused up to 95% mortality rates (S. Gomez-Jimenez unpubl. data).

Some comparative field data were collected of the unknown disease and white spot syndrome virus (WSSV), another devastating disease affecting shrimp culture within Sinaloa State. Altogether, 716 cases of the unknown disease were found versus 83 WSSV cases, representing 89.6 and 10.4%, respectively. A similar situation was also found in Sonora State, representing 81.1% of the unknown disease (137 cases) versus 18.9% of WSSV (32 cases). In terms of impact on production, the unknown disease caused a dramatic reduction in shrimp production within the affected regions. Production in the 3 states dropped by 57, 65 and 67% in Sinaloa, Sonora and Nayarit, respectively, compared with the shrimp production of 2012. Disease status was assessed by PCR assays for WSSV (S. Gomez-Jimenez unpubl. data).

At this point the causal agent was not known. Clinical signs were not similar to those of any known major shrimp diseases caused by either viruses or bacteria in NW Mexico prior to 2013. Shrimp affected by this unknown disease consistently showed the clinical signs of an atrophied hepatopancreas (HP) (shrunken, small and discolored) and an empty stomach and midgut. Additionally, mortalities appeared a few days after PL stocking into the ponds. All of the gross clinical signs of the affected shrimp were ominously suggestive of acute hepatopancreatic necrosis disease (AHPND) (NACA 2012).

In order to determine the cause of the mortalities a collaboration was started between the Centro de Investigación en Alimentación y Desarrollo, A.C. (CIAD) and the University of Arizona Aquaculture Pathology Laboratory (UAZ-APL). A recently developed PCR assay for detection of AHPND was used to prescreen *Vibrio* spp. isolates for the signature amplicon, designating an AHPND infection. Bacterial broth cultures that were positive by PCR were assessed using the challenge method developed by Tran et al. (2013) and by a modified challenge method in which the indicator *P. vannamei* were fed infected feed. Following the challenge trials with the suspected AHPND bacteria, histology was performed to confirm the infection.

Fig. 1. Northern states of Mexico, where high mortalities were registered during the 2013 shrimp culture cycle

MATERIALS AND METHODS

Fieldwork

Fieldwork was carried out during the 2013 shrimp farming season at commercial farms located in NW Mexico, particularly within the state of Sonora (Fig. 1). Most of these farms were presenting high mortalities at the time of sampling. Live shrimp from ponds presenting mortalities and from ponds without mortalities were transported in separated 400 l containers to the Marine Invertebrate Physiology Laboratory at Hermosillo, Sonora. Shrimp sampling was directed towards animals presenting abnormal gross signs according to technical staff from the farms. Such gross signs included a pale to whitish HP and a reduced size of this organ. Additionally, seawater and sediment from ponds were sampled.

Bacterial isolates

Two approaches were taken in order to obtain bacterial isolates. For the first approach the stomach, HP and mid-gut from the shrimp presenting gross signs of AHPND were aseptically removed, minced and separately inoculated into flasks containing tryptic soy broth with added 2% sodium chloride (TSB+). The flasks were incubated at 35°C for 18 to 24 h to obtain mixed cultures. All mixed cultures were subjected to sub-culture, in order to obtain individual colonies on the solid medium thiosulphate (TCBS) agar supplemented with 2.5% sodium chloride and incubated at 35°C overnight. Based on colony morphologies after incubation in TCBS agar plates, individual colonies were sub-cultured into alkaline peptone broth (APW) and streaked onto TCBS agar plates to confirm that a single isolate was obtained. All single isolates were preserved in TSB+ with 50% added glycerol and frozen at −80°C for further studies.

For the second approach, only the HP from shrimp were aseptically removed and minced. An aliquot was dropped and streaked on TCBS plates. Additionally, water and sediment samples were also streaked on TCBS plates. All samples were incubated at 35°C for 24 h. Unit-forming colonies were counted and subjected to sub-culture into APW and plated on TCBS, until pure isolates were obtained. All single isolates were preserved frozen as stated above.

Vibrio parahemolyticus strains

After pure cultures were obtained, all isolates were grown in APW for 18 to 24 h at 36°C before testing by PCR using primers specific for the thermostable hemolysin (*tlh*) gene as a marker for *V. parahaemolyticus* (Bej et al. 1999). All strains that were positive for *V. parahamolyticus* were then run by PCR for AHPND (see 'PCR' section).

Immersion bioassay

Using the isolation procedure described above, a bacterial isolate named 13-306D/4 was obtained from the HP of a Mexican shrimp specimen displaying gross signs of AHPND and was cultured overnight in TSB+ at 28°C. This was used to follow the immersion bioassay protocol described by Tran et al. (2013) in 2 replicate aquaria containing 5 *Penaeus vannamei* each. One positive control aquarium was used for treatment with the AHPND isolate 13-028A/3 previously obtained from Vietnam (Tran et al. 2013) and 1 negative control aquarium was for treatment with a non-pathogenic isolate of *V. parahaemolyticus* (13-028A/2). Following immersion, the shrimp were fed a pellet diet daily.

Per os bioassay

A second Mexican isolate (13-511A/1) isolated from pond sediment was used for bioassay tests with control isolates by per os exposure. After culture of bacterial isolates in TSB+ overnight at 28°C, a 1:1 ratio (wt/vol) of pelleted shrimp feed to broth culture was mixed for 10 min. The quantity of pelleted feed used was exactly 10% of the body weight of the 5 shrimp (~3 g = total weight 15 g) in each test aquarium. After the broth had soaked into the pellets, the entire mixture was used to feed the test shrimp in each appropriate aquarium. All subsequent feeding used normal, untreated pellets at 10% body weight per day. There were 2 replicate aquaria for the test isolate 13-511A/1 and 1 positive and 1 negative control aquarium each, again with isolate 13-028A/3 from Vietnam and non-pathogenic isolate 13-028A/2, respectively.

PCR

The PCR assay developed for the detection of *V. parahaemolyticus* AHPND-causing bacterial isolates targets a unique sequence that is present in only AHPND isolates. The forward primer 89F sequence, 5′-GTC GCT ACT GTC TAG CTG AA-3′ and the reverse primer 89R sequence 5′-ACG GCA AGA CTT AGT GTA CC-3′ amplify a 470 bp fragment using the following PCR cycling parameters: initial denaturation for 3 min at 95°C followed by 40 cycles at 95, 67 and 72°C for 20, 20 and 30 s, respectively. The cycling is completed with a final extension at 72°C for 3 min. The templates used in the PCR reactions were TSB+ inoculated with the various *V. parahaemolyticus* isolates discussed in this study. DNA was not extracted from the bacterial cultures. For more detailed information on the sensitivity and specificity of the PCR assay please refer to the Supplement at www.int-res.com/articles/suppl/d111p081_supp.pdf

Histology

Histopathology was initially the only method for determining AHPND infection. Samples from the bioassays that used the 2 Mexican AHPND-causing isolates were preserved in Davidson's AFA fixative, processed, and stained with hemotoxylin and eosin (Bell & Lightner 1988, Lightner 1996) for a confirmatory diagnosis.

RESULTS

The bacterial isolates were initially pre-screened using the PCR method. Fig. 2 shows the 470 bp amplicon present in the 3 AHPND-causing isolates (13-028A/3, 13-306D/4 and 13-511A/1). The *Vibrio parahaemolyticus* isolate (13-028A/2) that did not cause AHPND by immersion challenge when used as the template for PCR did not result in an amplified product (Fig. 2).

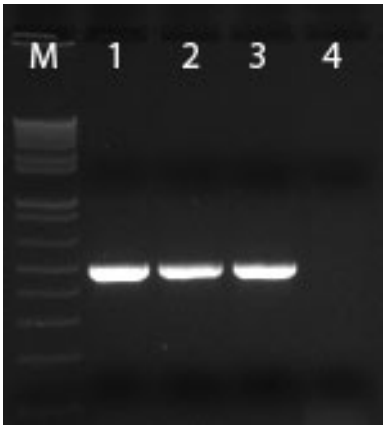

Fig. 2. PCR amplification of the 470 bp amplicons for detection of acute hepatopancreatic necrosis disease (AHPND) using primer 89F/R. Lanes: M, 1 Kb Plus DNA marker (Invitrogen/Life Technologies); 1, 13-306D/4; 2, 13-511A/1; 3, 13-028A/3; 4, 13-028A/2

The initial challenge method was by immersion (Tran et al. 2013). Fig. 3A graphically represents the survival data from the experiment. Within 4 d 100% mortality resulted in the shrimp that were immersed in AHPND-causing *V. parahaemolyticus* broth and this contrasted with the non-AHPND-causing isolate that results in 100% survival of the challenged *Penaeus vannamei*. The results from the second challenge method, per os feeding of bacterial soaked pelleted shrimp feed, are shown in Fig. 3B. This challenge experiment, with the bacteria isolated from pond sediment, resulted in 100% mortality within 3 d. Survival in the negative control group fed broth-soaked pellets was 90% (Fig. 3B).

Histological analysis of moribund shrimp samples from the immersion and per os bioassays revealed the presence of AHPND lesions (Fig. 4). These lesions were characterized by acute sloughing of hepatopancreatic tubule epithelial cells, some of which displayed intact organelles such as nuclei and cytoplasmic vesicles. The epithelial cells that remained attached to the tubules appeared dysfunctional, and mild hemocytic congestion could be observed within the intertubular spaces (Fig. 4). No bacterial colonization was evident within the HP, but bacterial plaques were found within the stomach chambers in some of the affected shrimp (not shown).

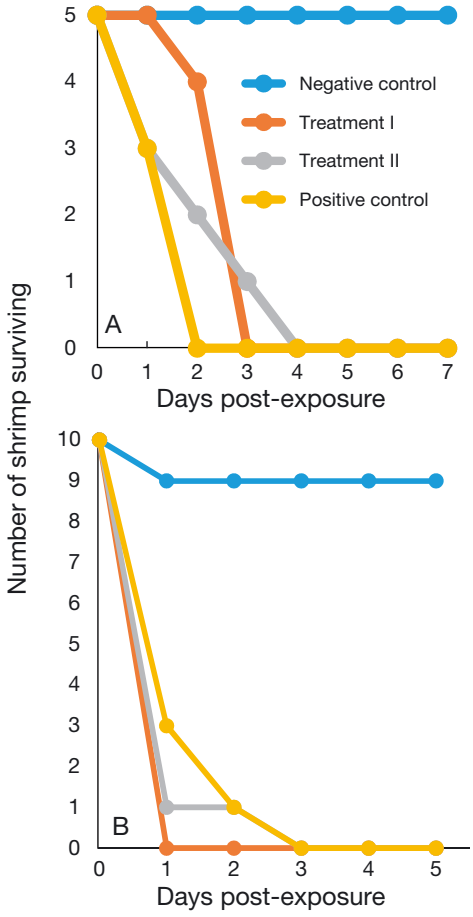

Fig. 3. (A) Results from the immersion treatment that tested *Vibrio parahaemolyticus* isolate 13-306D/4, isolated from the hepatopancreas. (B) Results from the per os treatment that tested *V. parahaemolyticus* isolate 13-511A/1, isolated from pond sediment

DISCUSSION

Vibrio parahaemolyticus is ubiquitous in the marine environment. Both pathogenic and benign strains exist. The isolates that cause AHPND are extremely virulent. With the intensification of shrimp farming worldwide, new pathogens seem to emerge frequently. The first step in preventing the spread of a newly recognized disease is to identify the causal agent. Once the pathogen has been determined, the next priority is the development of a diagnostic test to identify or detect the pathogenic agent. Testing by PCR has become a standard for screening of major diseases in cultured shrimp, including viruses, bacteria and parasites that may be causing disease in ponds.

A rapid PCR test that was developed for the detection of AHPND was used in this study to confirm the presence of the pathogen in broth cultures that originated from Mexico. The PCR test is able to distinguish between pathogenic AHPND-causing *V. parahaemolyticus* isolates and non-pathogenic

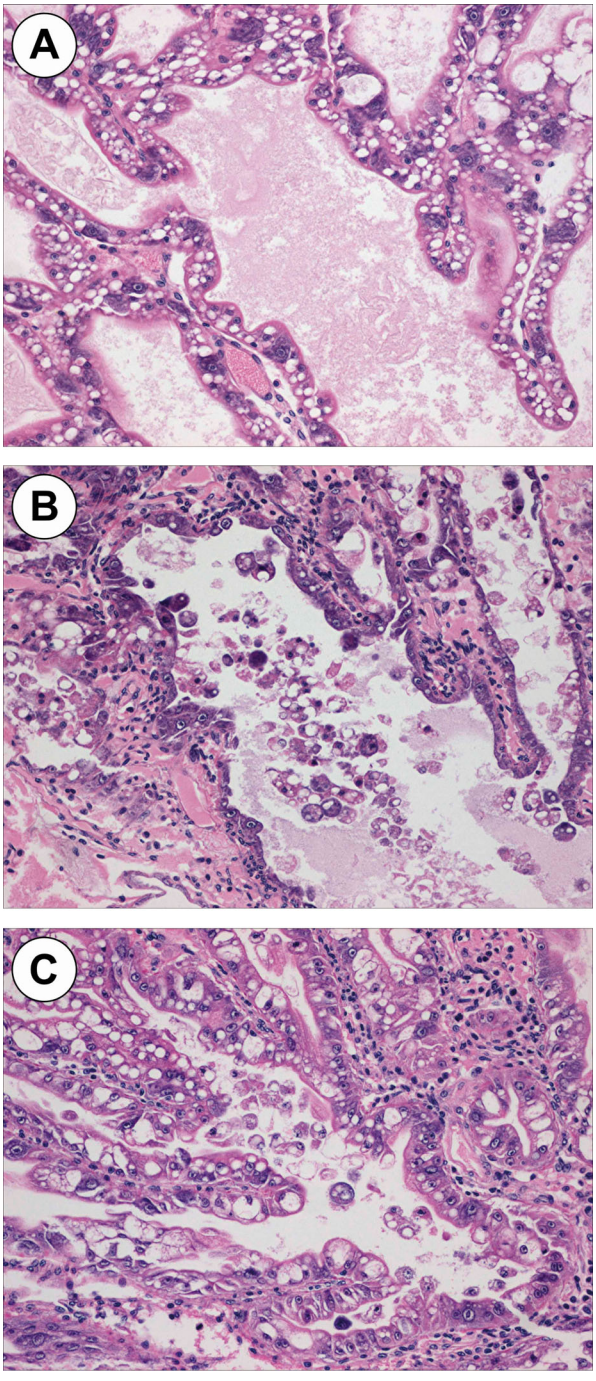

Fig. 4. Histology results from the per os and immersion bioassays to test the pathogenicity of *Vibrio parahaemolyticus* isolates obtained from hepatopancreas and from sediment. (A) Negative control. Normal appearance of the hepatopancreas. (B) Acute hepatopancreatic necrosis disease (AHPND) from per os bioassay. Notice the acute sloughing of hepatopancreas tubule epithelial cells. (C) AHPND from immersion bioassay. The acute sloughing of hepatopancreas tubule epithelial cells is evident. In both cases (B & C), there is no obvious bacterial colonization within the tubules, and epithelial cells appear dysfunctional. Stain: Mayer-Bennett's hematoxylin/eosin-phloxine. Total magnification: 50×

isolates. This test can help the industry by providing a method that allows AHPND screening of broodstock and post larvae prior to stocking ponds. In addition, the PCR test will allow determination of the presence of AHPND in ponds suffering high mortalities in juveniles soon after stocking into ponds.

This study was undertaken to confirm the presence of AHPND in Mexico. Using PCR, the causative bacterium was tentatively identified. Challenge studies were conducted to investigate if the identified bacterium would cause mortalities in a laboratory environment. Finally, histology was employed to confirm that the pathology present in challenged *Penaeus vannamei* was indeed caused by *V. parahaemolyticus*, the agent that causes AHPND. The culmination of these methods was the confirmation that the mortalities that occurred from March until June in 2013, in 3 northern Mexican states, were caused by AHPND, which is this first report of this pathogen in the Western Hemisphere.

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LITERATURE CITED

Bej AK, Patterson DP, Brasher CW, Vickery MCL, Jones DD, Kaysner CA (1999) Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh*, and *trh*. J Microbiol Methods 36:215–225

Bell TA, Lightner DV (1988) A handbook of normal shrimp histology. Special Publication No. 1. World Aquaculture Society, Baton Rouge, LA

de Schryver P, Defoirdt T, Sorgeloos P (2014) Early mortality syndrome outbreaks: a microbial management issue in shrimp farming? PLOS Pathogens 10:e1003919

FAO (Fisheries and Agriculture Organization) (2013) Report of the FAO/MARD technical workshop on early mortality syndrome (EMS) or acute hepatopancreatic necrosis syndrome (AHPNS) of cultured shrimp (under TCP/VIE/3304). Report No. 1053. FAO, Rome

Leaño EM, Mohan CV (2012) Early mortality syndrome (EMS)/acute hepatopancreatic necrosis syndrome (AHPNS): an emerging threat to the Asian shrimp industry. NACA, Bangkok

Lightner DV (1996) A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp. World Aquaculture Society, Baton Rouge, LA

NACA (Network of Aquaculture Centres in Asia Pacific) (2012) Diseases of crustaceans — acute hepatopancreatic necrosis syndrome (AHPNS). This disease card was prepared based on the outcomes of the Asia Pacific Emergency Regional Consultation on Early Mortality Syndrome (EMS)/Acute Hepatopancreatic Necrosis Syn-

drome (AHPNS) held in Bangkok, Thailand, on 9–10 August 2012. 5m Publishing, Sheffield

The Fish Site (2013) Fighting EMS shrimp disease in Mexico. Available at: www.thefishsite.com (accessed 4 Feb 2013)

Tran L, Nunan L, Redman RM, Mohney LL, Pantoja CR, Fitzsimmons K, Lightner DV (2013) Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Dis Aquat Org 105:45–55

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