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Short communication

Evaluation of a reliable non-invasive molecular test for the diagnosis of the causative agent of acute hepatopancreatic necrosis disease of shrimp

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ABSTRACT

Acute hepatopancreatic necrosis disease (AHPND, also known as early mortality syndrome, EMS) has caused substantial mortality, up to 100%, in populations of penaeid shrimp cultured in SE Asia and in Latin America. The disease is caused by the bacterium *Vibrio parahaemolyticus*, which secretes binary toxins ($PirA_{vp}$ and $PirB_{vp}$) resulting in the deterioration of the hepatopancreas tissue of infected shrimp. Diagnosis, screening, and monitoring of AHPND in shrimp populations involve sacrificing individuals to obtain tissue samples. This sampling method is undesirable when applied to valuable populations of broodstock. Here, we evaluated a non-invasive diagnostic method based on shrimp fecal samples that are analyzed by PCR. Small groups of Pacific white shrimp *Penaeus vannamei* were exposed to low levels of AHPND-bacteria and their feces were collected prior to any mortality observed (in the bioassays #1 and #2). Two protocols were evaluated. In one, DNA extracted from the fecal samples was directly analyzed by PCR. In the other, the fecal samples were cultured in TSB+ for 6 h to enrich the bacterial populations, then the enriched bacterial broth was used for PCR analyses. Our results showed that the presence of *V. parahaemolyticus* could be detected both in fecal DNA samples and in the enriched bacterial broth, but the bands from the bacterial broth showed stronger amplification than the DNA; 12 strong positive in the enriched bacterial broth, but only 7 strong positive in the fecal DNA samples. Also, the AHPND bacteria present in the feces is infectious, determined by a bioassay of feeding specific pathogen free indicator shrimp with AHPND-feces (in the bioassay #3), and this proves that the AHPND can be transmitted through a fecal-oral route.

1. Introduction

Acute hepatopancreatic necrosis disease (AHPND) is a major enteric disease of penaeid shrimp (Flegel, 2012; Leano and Mohan, 2012; Lightner et al., 2012) caused by specific strains of *Vibrio parahaemolyticus* (Tran et al., 2013). The infection resulted in the sloughing and necrosis of hepatopancreas and leads to high mortalities, up to 100%, in affected populations (Yang et al., 2014; Han et al., 2015a; Sirikharin et al., 2015; Lee et al., 2015). This disease has been detected in shrimp cultured in SE Asia and Latin America since 2009 (Han et al., 2015a,b).

AHPND only infects tissue of the hepatopancreas, thus, its diagnosis usually involves sacrificing the shrimp to collect tissue samples for analysis via histology and/or PCR. This sacrificial method is obviously not desirable for monitoring valuable broodstock populations. Alternatively, because the AHPND bacteria are present in the digestive systems of infected shrimp, fecal samples can be taken for PCR analysis in the monitored populations without causing mortality. This non-invasive method has been used successfully for the diagnosis and monitoring of other enteric pathogens of shrimp, including hepatopancreatic parvovirus (HPV), necrotizing hepatopancreatitis (NHP-B), and *Enterocytozoon hepatopenaei* (EHP) (Pantoja and Lightner, 2000; Vincent and Lotz, 2005; Tang et al., 2015).

In this study, a non-invasive PCR method was evaluated using shrimp fecal samples for the AHPND diagnosis. This finding will be helpful for monitoring of valuable broodstock populations, and also developing of strategies for disease management.

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2. Materials and methods

2.1. Bacteria preparation

The AHPND-pathogenic strain of *V. parahaemolyticus* 13-028/A3 originated from Vietnam was used in this study. The pathogenicity of the strain was previously determined by laboratory infections and followed by histological examinations (Tran et al., 2013). Its AHPND toxin genes were analyzed through whole genome sequencing (Han et al., 2015a). This strain was cultured in TSB+ (Tryptic soy broth plus 2% NaCl) with gentle shaking (100 rpm) at 28–29 °C for 20 h, and used for AHPND infection of shrimp.

2.2. Experimental shrimp and water tanks

Two populations of specific-pathogen free (SPF) shrimp (*Penaeus vannamei*) were used: 6 large juveniles (weights: 8–9 g) for the bioassays #1, and 180 small juveniles (weights: 0.5–1.0 g) for the bioassays #2 and #3. Large juveniles were individually maintained in 3-L tanks with aeration (at a salinity of 25 ppt and water temperature at 25–28 °C), and small juveniles (N = 20) were maintained in 90-L tanks equipped with submerged biological filters (White et al., 2002). Shrimp were fed shrimp feed, Rangen 35% (Buhl, ID) at a total of 5% bodyweight each day for the duration of the study.

2.3. Disinfecting solutions

To disinfect shrimp, 2 solutions (20-L each) were prepared according to modifications of the guidelines from Underwood (1990) and Alday-Sanz (2010): (1) 300 ppm formalin (0.3 mL of 37% formaldehyde in 1-L artificial salt water at 25 ppt salinity) and (2) 100 ppm povidone-iodine (0.1 mL of povidone-iodine stock solution in 1-L artificial salt water at 25 ppt salinity).

2.4. Infection tests

Three shrimp bioassays were conducted in this study. The bioassay #1 was designed for testing the potential amplification of AHPND from fecal samples of large juveniles. The bioassay #2 was designed to support data from the bioassay #1 using small juveniles. The collected fecal samples were further used for the broth enrichment method to increase the detection limit, and the AHPND infection in the bioassay #3. The bioassay #3 was designed to determine the infectivity of the feces.

2.4.1. Feces collection from large juveniles (Bioassay #1)

In the bioassay #1, 4 SPF shrimp (weights: 8–9 g) were exposed to the AHPND bacterial culture by *per os* feeding method in a 90-L tank. Briefly, feed pellets (at a total of 5% bodyweight) were soaked in AHPND-bacteria culture (10^8 CFU/mL) at 1:1 ratio for 5 min, and shrimp were fed AHPND-feed. After 3 h, all 4 shrimp were taken out using a hand net, and submerged in a formalin solution (300 ppm) for 30 s, then submerged in a povidone-iodine solution (100 ppm) for 1 min, rinsed in clean seawater, and finally placed in the 3-L tanks individually with aeration.

During the bioassay (3 days), shrimp were observed every 6 h, and fed with normal shrimp feed. Fecal strands (2 g) were collected from the bottom of the tanks during the bioassays, pooled and stored at −20 °C until used for the DNA extraction. For negative controls, 2 SPF shrimp were not challenged with AHPND-*V. parahaemolyticus* and fecal strands were collected.

2.4.2. Feces collection from small juveniles (Bioassay #2)

In the bioassay #2, 80 SPF shrimp (weights: 0.5–1.0 g) were stocked in 90-L tanks (2 tanks, 40 shrimp/tank), and exposed to the AHPND bacterial culture by immersion method at a concentration of 10^6 CFU/mL water. After 6-h immersion, 40 shrimp were taken out from the tanks and stocked in the clean 90-L tanks equipped with a submerged biological filter 90-L tanks, followed by disinfecting methods described above. For lower levels of AHPND infection, 40 shrimp were moved after 1-h immersion with AHPND bacterial culture, disinfected, and placed in the cleaned 90-L tanks.

During the bioassay (6 days), fecal strands (2 g) were stored as described above for the DNA extraction. Non-frozen fecal samples (2 g each) were further used for the broth enrichment method and the AHPND infection in the bioassay #3. For negative control, 20 SPF shrimp were not challenged with AHPND-*V. parahaemolyticus* and fecal strands were collected.

2.4.3. AHPND infection through a fecal-oral route (Bioassay #3)

In the bioassay #3, 40 SPF shrimp (weights: 0.5–1.0 g) were stocked in 290-L tanks, and exposed to the AHPND-feces homogenate by *per os* feeding method. Briefly, AHPND-feces were collected from the bioassay #2, and homogenized with 25 ppt seawater (2 g/2 mL). Then, regular shrimp feed pellets were soaked in AHPND-feces homogenates (1:1 ratio) for 5 min, and fed to shrimp at a 5% bodyweight for the duration of the study (4 days).

Three hours after feeding, feed and feces were removed from each tank, and 5 shrimp from each tank were sampled every day and frozen at −20 °C. Also, at the termination day, fecal strains were collected from each tank. As negative controls, shrimp (N = 40) were maintained in 290-L tanks, and fed with feed pellets soaked in SFP-feces homogenates, and fecal strands were collected.

2.5. DNA extraction and AHPND PCR assays

Fecal strands collected from the bioassay #1, #2 and #3, and hepatopancreas samples from the bioassay #3 were used for the DNA extraction procedure using a DNeasy blood and tissue kit (Qiagen) or a Maxwell-16 ® Cell LEV DNA purification kit (Promega). Then, extracted DNA was used as a template for the AHPND PCR assays (Han et al., 2015a).

2.6. Broth enrichment and AHPND PCR assays

To increase the detection limit, fecal strands collected from the bioassays #2 and #3 were used for the broth enrichment procedure modified from the methods of Kongmuang et al. (1994) and Rahn et al. (1992). Fecal strands were homogenized, 20 mg of feces homogenate was inoculated in 20 mL of TSB+, and cultured at 28–29 °C with gentle shaking (100 rpm) for 6 h. Then, the enriched bacterial broth (1 μL) was used directly as a template for the AHPND PCR assays.

3. Results and discussion

3.1. AHPND detection in fecal samples from the bioassay #1

We applied the AHPND PCR analyses using fecal samples for non-invasive screening of AHPND. In the bioassay #1, large juveniles (8–9 g) were orally challenged by feeding them with feed pellets soaked in a sub-lethal suspension, and 10^8 CFU/mL was successful for obtaining infected but asymptomatic shrimp. Among 4, only 1 shrimp (shrimp #4) died at day 2, and 3 shrimp that challenged by AHPND were asymptomatic at the termination day (Table 1).

Prior to the PCR testing, DNA extraction was performed for the fecal samples to eliminate any potentially interfering materials. It is reported that the bilirubin and bile salts present in the feces might inhibit PCR amplification (Tada et al., 1992; Widjojoatmodjo et al.,