

Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp

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ABSTRACT: A new emerging disease in shrimp, first reported in 2009, was initially named early mortality syndrome (EMS). In 2011, a more descriptive name for the acute phase of the disease was proposed as acute hepatopancreatic necrosis syndrome (AHPNS). Affecting both Pacific white shrimp *Penaeus vannamei* and black tiger shrimp *P. monodon*, the disease has caused significant losses in Southeast Asian shrimp farms. AHPNS was first classified as idiopathic because no specific causative agent had been identified. However, in early 2013, the Aquaculture Pathology Laboratory at the University of Arizona was able to isolate the causative agent of AHPNS in pure culture. Immersion challenge tests were employed for infectivity studies, which induced 100% mortality with typical AHPNS pathology to experimental shrimp exposed to the pathogenic agent. Subsequent histological analyses showed that AHPNS lesions were experimentally induced in the laboratory and were identical to those found in AHPNS-infected shrimp samples collected from the endemic areas. Bacterial isolation from the experimentally infected shrimp enabled recovery of the same bacterial colony type found in field samples. In 3 separate immersion tests, using the recovered isolate from the AHPNS-positive shrimp, the same AHPNS pathology was reproduced in experimental shrimp with consistent results. Hence, AHPNS has a bacterial etiology and Koch's Postulates have been satisfied in laboratory challenge studies with the isolate, which has been identified as a member of the *Vibrio harveyi* clade, most closely related to *V. parahemolyticus*.

KEY WORDS: AHPNS · Early mortality syndrome · EMS · *Penaeus monodon* · *Penaeus vannamei* · Etiology · *Vibrio*

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INTRODUCTION

The shrimp farming region of Southeast Asia and China supports the largest and most productive shrimp farming industry in the world. Beginning in about 2009, a new, emerging disease called 'early mortality syndrome' or 'EMS' (more descriptively called acute hepatopancreatic necrosis syndrome or AHPNS; Lightner et al. 2012) began to cause significant production losses in southern China (NACA-

FAO 2011). By 2010, the range of affected farms in China had expanded, and by 2011, AHPNS was confirmed in Vietnam and Malaysia (Lightner et al. 2012, Mooney 2012). EMS disease reached Thailand in 2012 (Flegel 2012, Leño & Mohan 2012). As has been the case with other epizootic shrimp diseases (Stentiford et al. 2012), EMS is causing serious production losses in affected areas and is also impacting employment, social welfare, and international markets (Bondad-Reantaso et al. 2012, Mooney 2012).

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Since its first emergence, the etiology of AHPNS remained unknown. Initial laboratory studies failed to demonstrate an infectious etiology. The pathology of AHPNS is limited to the hepatopancreas (HP), and the nature of that pathology suggested that the disease had a toxin-mediated etiology (Lightner et al. 2012). The gross signs of AHPNS are evident by pond-side examination of affected shrimp accompanied by dissection and examination of the HP. These signs may become apparent as early as 10 d post-stocking in a recently prepared pond. Shrimp with early AHPNS will show a pale to white HP due to pigment loss in the HP R-cells, as well as atrophy of the HP that may reduce the expected size of the organ by 50% or more. In the terminal phase of the disease, black streaks or spots (due to melanin deposition from hemocyte activity) appear in the HP.

Following routine histological methods (Lightner 1996), the histopathology of AHPNS presents as an acute progressive degeneration of the HP from proximal to distal with dysfunction of R-, B-, F-, and E-cells. Affected HP tubule epithelial cells often display prominent karyomegaly. Such cells round-up and detach from the affected HP tubules, and become necrotic within the HP tubules or in the gut lumen. In the terminal phase of AHPNS, the HP shows marked intra- and inter-tubular hemocytic infiltration and development of massive secondary bacterial infections that occur in association with the necrotic and sloughed HP tubule epithelial cells. Lesions found in infected individuals of both Pacific white shrimp *Penaeus vannamei* and black tiger shrimp *P. monodon* are identical (Lightner et al. 2012).

Preliminary studies to determine the cause of AHPNS

Two approaches were initially attempted by the Aquaculture Pathology Laboratory at the University of Arizona (UAZ-APL) to determine the etiology of AHPNS. These included studies aimed at: (1) finding an environmental toxin as the possible cause of the disease and (2) testing for infectious agent(s). Included among the studies in (1) (environmental toxins) were water and sediment samples, algae from AHPNS-affected ponds, feed samples (because of the potential for mycotoxin) from affected farms, and pesticides used for killing white-spot syndrome virus vectors. None of these studies was successful at reproducing the pathology characteristic of AHPNS (Lightner et al. 2012).

Under point (2) (testing for an infectious agent), several infectivity studies were conducted at UAZ-APL using frozen materials collected from affected farms in Vietnam in 2011 and 2012. A number of infectivity methods were employed in an effort to reproduce AHPNS lesions in experimentally challenged shrimp. The methods included: intramuscular (IM) injections with 0.45 μm filtered and unfiltered inocula and *per os* (feeding experimental shrimp with AHPNS-infected shrimp carcasses). None of these methods was found to induce pathology of the HP consistent with AHPNS (Lightner et al. 2012).

Infectivity studies conducted in Vietnam

Because AHPNS was suspected to be caused by a biological agent, UAZ-APL had concerns that the potential infectious agent in frozen materials might have been inactivated by freezing and thawing. Another concern was that the infection models, by IM injection conducted at UAZ-APL in preliminary studies, might not be the most natural route of infection for AHPNS. Based on those preliminary studies, the UAZ-APL conducted several on-site studies in an AHPNS endemic area in the Mekong Delta of Vietnam during mid- to late 2012. Several challenge methods were used in which only live or fresh materials (never frozen) from AHPNS-affected shrimp were tested for pathogenicity. In July 2012, IM injection with filtered and unfiltered AHPNS-infected shrimp homogenates, reverse gavage (Aranguren et al. 2010), *per os*, and co-habitation studies were conducted. The results from these studies showed that AHPNS lesions could be induced in experimental shrimp in *per os* and co-habitation studies. These results were confirmed by routine histological methods as described by Bell & Lightner (1988) and Lightner (1996).

Histological examination of infected shrimp consistently shows that the AHPNS pathology is limited to the HP and that significant bacterial involvement does not appear within the HP during the acute phase of AHPNS. Based on the progression of the pathology, and the suggested nature of the infection route, as determined by the infectivity studies, we worked on the hypothesis that the causative agent(s) may colonize the shrimp digestive tract in the early stage of infection and that those bacterial colonies might be able to produce a toxin(s) capable of causing HP dysfunction.

In December 2012, another on-site study was conducted in the Mekong Delta of Vietnam with

the intent to focus on bacterial populations found in the stomach and HP of infected shrimp collected in Vietnam and not frozen. Two trials with a mixture of bacteria isolated from the stomach and HP of AHPNS-infected shrimp were run. With each mixed culture of bacteria, 2 challenge studies were conducted using either bacteria growing on solid media (tryptic soy agar with added 2% sodium chloride, TSA+) fed to experimental shrimp, or bacteria grown in liquid media (tryptic soy broth with added 2% sodium chloride, TSB+) for the immersion study. Of these treatments, only the immersion treatment with mixed stomach bacteria was found to induce AHPNS-typical lesions, which were confirmed by histological examination (unpubl. data). Based on the result that the mixed culture of infected shrimp stomach bacteria could induce AHPNS pathology, the same mixed culture was brought back from Vietnam for further studies conducted at UAZ-APL.

MATERIALS AND METHODS

Bacterial isolates

In Vietnam in December of 2012, the stomachs from AHPNS-positive shrimp were aseptically removed, minced, and separately inoculated into flasks containing 30 ml of TSB+; the flasks were then incubated at 28°C for 18 h to obtain mixed cultures. The mixed bacterial cultures found to induce AHPNS by immersion experiments in Vietnam were preserved in TSB+ with 25% added glycerol, frozen in dry-ice, and stored at -20°C prior to being transported frozen for experiments at UAZ-APL. Once at UAZ-APL, the mixed cultures of interest were subjected to sub-culture to obtain individual colonies and pure cultures on TSA+ plates. The only mixed-bacteria culture used in the experiments at UAZ-APL was designated as mixed culture A, and 3 sub-cultures of mixed culture A, designated as A/1, A/2, and A/3, were also tested for their pathogenicity in the experiments. After pure cultures were obtained, bacterial identifications were conducted by using API Rapid NE test and 16S rRNA sequencing.

Media types

Both solid and liquid media were used for bacterial culture. The solid media used were TSA+, Marine

Agar™ (Difco), and TSA+ with added 5% sheep blood (Blood Agar) in an attempt to isolate the causative agent of AHPNS. The liquid medium used to replicate the results from Vietnam was TSB+.

Immersion challenge tests

Both liquid and solid media were employed to grow the bacterial samples (both mixed and pure bacterial cultures) for inoculum preparation for challenge tests. For liquid media, the inocula were prepared by separately inoculating the glycerol-preserved bacterial isolates into flasks containing 30 ml of sterile TSB+, and then the flasks were placed in the rotary shaker and incubated for 18 h at 28°C. After 18 h of incubation, TSB+ solutions were checked using a spectrophotometer at OD₆₀₀ to determine bacterial density. Inocula grown on solid media were prepared as follows: the glycerol preserved isolates were inoculated in TSB+ for 4 h at 28°C in the rotary shaker before plating onto 3 different solid media (TSA+, marine agar, and sheep blood agar) and incubating for 18 h at 28°C. Bacteria grown on solid media were scraped, and then re-suspended in saline water for immersion experiments. The immersion procedure was carried out by immersing 15 shrimp for 15 min with aeration in a flask containing a solution of approximately 150 ml prepared by mixing of either TSB+ broth culture or the bacterial suspension obtained from bacteria grown on solid agars with saline water to achieve a bacterial density of approximately 2×10^8 cells ml⁻¹. Following the 15 min immersion in the bacterial suspension, this same suspension was added directly into an experimental tank containing clean artificial seawater to obtain an approximate bacterial density of 2×10^6 cells ml⁻¹, and then the immersed animals were transferred into the experimental tank. Shrimp in the negative control group were immersed in sterile TSB+.

Reverse gavage challenge test

The reverse gavage challenge tests were conducted following the method described by Aranguren et al. (2010). The broth medium, after being inoculated with bacteria and incubated for 18 h, was centrifuged at 6000 rpm (3200 × g) (5 min). The supernatant fluid was filtered through a 0.2 µm filter. Food colorant was added to filtered supernatant fluid

before being delivered to the shrimp. Each experimental shrimp in the reverse gavage treatment received 2 doses of 0.1 ml of the filtered supernatant through the anal route on Days 0 and 2. The negative control shrimp received 2 doses of 0.1 ml of sterile TSB+ via reverse gavage on Days 0 and 2 of the experiment.

Indicator animals

Animals used in the experiments were Kona line specific-pathogen free (SPF) Pacific white shrimp *Penaeus vannamei* (Lightner et al. 2009, Moss et al. 2012). The experimental shrimp ranged in weight from 0.5 to 2 g.

Tank preparation

Depending on the experimental design, either 90 l tanks or small 4 l glass jars were used. Each tank or jar was equipped with a submerged biological filter and filled with artificial seawater at a salinity of 25 ppt. Water temperature was maintained at around 26 to 28°C, dissolved oxygen was maintained above 5 ppm, and total ammonia concentration was kept below 0.1 ppm. Experimental tank set-up followed the methods described by White et al. (2002).

Expt 1: Immersion study with mixed bacteria isolated from AHPNS-positive shrimp

Four 90 l plastic tanks were employed for 3 replications of the immersion treatment with the mixed bacteria isolated from infected shrimp stomach (mixed culture A) that previously had been shown to induce AHPNS in shrimp by immersion challenge tests conducted in Vietnam. One negative control tank was included. Each tank was stocked with 15 SPF *Penaeus vannamei*.

Expt 2: Immersion challenge study with individual bacterial isolates

Three pure cultures (A/1, A/2, and A/3), isolated from mixed culture A, were tested for pathogenicity. All 3 pure cultures were grown in TSB+ for inoculum preparation for immersion challenge tests. The positive control group was treated with the known, consistently pathogenic, mixed bacterial culture A, also

grown in liquid media (TSB+). Sterile TSB+ was used for negative control in the challenge. In addition, mixed culture A was also grown on 3 different solid agars (TSA+, marine agar, and sheep blood agar), for an additional immersion challenge test. This experiment was carried out in 4 l jars containing 5 experimental shrimp each. Three replications were used for each treatment.

Expt 3: Immersion bioassay with bacteria isolated from AHPNS-positive treatments from Expt 2

The results obtained from Expt 2 indicated that only the A/3 colony from the shrimp stomach could cause AHPNS pathology in SPF shrimp. In addition, the same type of colony was also recovered from the water from the AHPNS-positive tank in which shrimp were treated with mixed culture A. This isolate was named B/1. The purpose of Expt 3 was to repeat the immersion experiment with the pure culture of bacteria re-isolated from the infected shrimp stomach from Expt 2 in order to prove that AHPNS is a true infectious disease (Lightner 1988, Hasson et al. 1995), and to complete Koch's Postulates (Lightner 1988, Saulnier et al. 2000, Hasson et al. 2009). In addition, because the results of Expt 2 indicated that AHPNS could not be introduced by immersion treatments with bacteria grown on solid agars, a reverse gavage study was conducted using the filtered supernatant fluid from TSB+ that was inoculated with A/3. An immersion treatment with the known pathogenic isolate, the mixed culture A, served as a positive control. Negative control shrimp were treated with sterile TSB+ via reverse gavage. This experiment was carried out in 4 l jars containing 5 experimental shrimp each. Three replications were applied for each treatment.

Observation and sampling

Shrimp were fed twice daily with a pelleted shrimp feed (35% protein, Rangen) for 5 d. During the bioassay period, shrimp were checked approximately every 6 h. Dead shrimp were removed from the experimental tanks and frozen at -70°C. When observed, 1 to 2 moribund shrimp were fixed in Davidson's AFA fixative for histology (Bell & Lightner 1988, Lightner 1996). At termination (5 d), 2 to 3 shrimp from each tank were fixed for histology while some were used for re-isolation of bacterial cultures.

Histopathology

All shrimp sampled for histopathology purposes were injected with AFA Davidson's fixative, processed, and stained with hematoxylin and eosin-phloxine (H&E) using routine histological methods described by Lightner (1996). The histological sections were analyzed by light microscopy for AHPNS/EMS lesions in the HP. Lesion severity was graded accordingly to the G-grading system (Lightner 1996), from G0 for negative to G4 as the highest severity of AHPNS/EMS.

RESULTS

Expt 1: Immersion study with mixed bacteria isolated from AHPNS-positive shrimp

Shrimp immersed in the bacterial broth containing mixed culture A began to develop AHPNS gross signs, and the first mortality occurred within 18 h. Mass mortalities occurred from 18 h post-challenge until Day 4, when the experiment was terminated because cumulative mortalities had reached 100% (Fig. 1). Gross signs presented by the challenged *Penaeus vannamei* included an empty gastrointestinal tract, whitish, 'milky' appearance of the stomach, whitish, atrophied HP, lethargy, and soft shells (Fig. 2A,B).

Upon routine histological examination (Lightner 1996) using the G-grading system, negative control shrimp HPs appeared normal with normal structure of tubules and epithelial cells (Fig. 3A,B). In contrast,

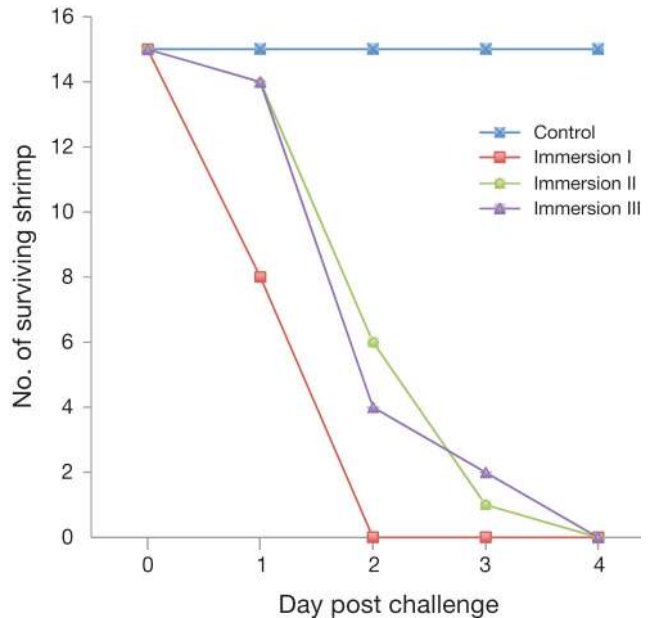


Fig. 1. *Penaeus vannamei*. Mortalities induced in the immersion bioassay using the mixed bacterial culture

after 48 h, the HPs of shrimp treated with the mixed bacteria culture showed G2–3 HP tubule cell sloughing, G0–1 B-cells, virtually no R-cells, G0–1 F-cells, some development of nuclear karyomegaly of the HP tubule cells, some hemocytic infiltrates, and virtually no bacterial colonization. This was scored as an overall G3–4 AHPNS (Fig. 3C,F). After 72 h of exposure to the agent, AHPNS-infected shrimp HPs exhibited G0 lipids, G0 B-, F-, and R-cells, G3–4 sloughing, G3–4 hemocytic infiltration and bacterial colonization, and an overall G4 AHPNS/EMS (Fig. 3D,E).

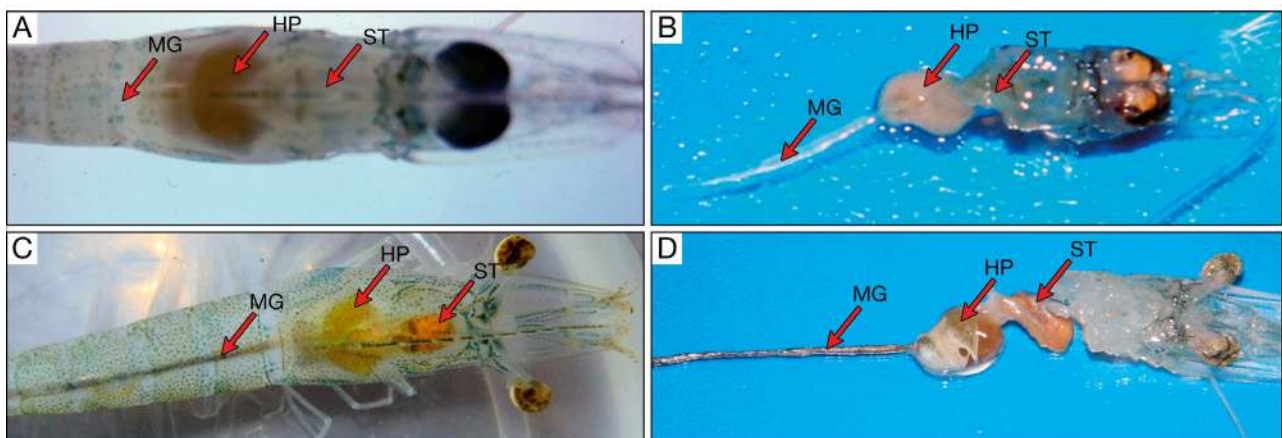


Fig. 2. *Penaeus vannamei*. (A,B) Gross signs of AHPNS-infected shrimp. Pale, atrophied hepatopancreas (HP), and an empty stomach (ST) and midgut (MG), which was induced by immersion bioassay. (C,D) Normal shrimp in the negative control group, showing a normal size HP with dark orange color and a full stomach and midgut. (B) and (D) are dissected individuals from (A) and (C), respectively

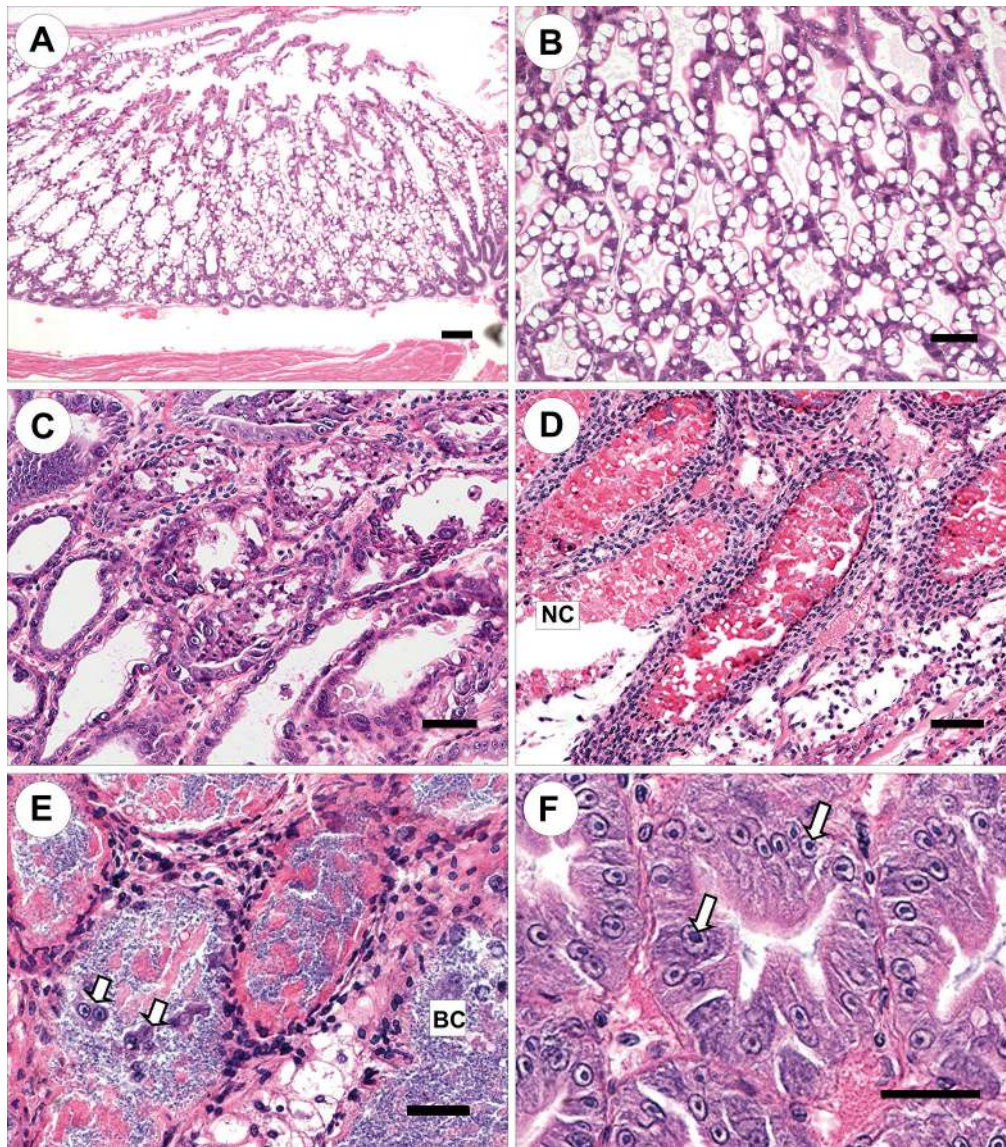


Fig. 3. *Penaeus vannamei*. Histological sections from the immersion bioassay. (A,B) Normal shrimp from the negative control group showing normal structure. (C–F) Lesions induced by immersion treatments with mixed culture A showing: (C) acute phase with no B-, F-, and R-cells, and sloughing of hepatopancreas (HP) tubule epithelial cells; (D) early terminal phase: necrotic sloughing of HP tubule epithelial cells (NC) and the remnants of HP tubules which are surrounded by hemocytic infiltration; (E) terminal phase: sloughing cells (arrows) are shown in the HP tubule lumens along with significant bacterial colonization (BC), and remnants of HP tubules are surrounded by hemocytic infiltrates; and (F) affected tubules with no B-, F-, and R-cells, and some nuclei (arrows) of HP tubule epithelial cells are enlarged (karyomegaly). Scale bars = (A,B,D) 100 μ m, (C,E) 50 μ m, (F) 30 μ m

The result from Expt 1 indicated that the mixed bacterial culture isolated from AHPNS-infected shrimp from Vietnam induced the same AHPNS pathology as described by Lightner et al. (2012) in SPF shrimp by immersion challenge. The results of Expt 1 reinforced the results of the immersion infectivity study conducted in Vietnam (UAZ-APL, unpubl. data) showing that the mixed bacterial culture from

the stomach of infected shrimp induced AHPNS pathology during the immersion challenge study.

Expt 2: Immersion challenge studies with individual bacterial isolates

The resulting AHPNS typical gross signs and mortalities presented by the challenged *Penaeus van-*

namei indicated that the type of media used may affect the ability of mixed culture to induce the pathology in experimental shrimp (Table 1, Expt 2). Although the bacterial densities in all immersion treatments were adjusted to 2×10^8 cells ml⁻¹, the different media types used to grow the bacteria gave different results. The immersion treatment with mixed culture A grown in liquid media (TSB+) caused typical AHPNS gross signs and mortalities in experimental shrimp within 18 h after exposure. Upon histological examination of fixed samples, 100% of the samples in the TSB+ treatment were positive for AHPNS pathology at high severity grades (G3–4). In contrast, all treatments with the same mixed culture A grown on solid media did not induce any AHPNS gross signs until 3 d after exposure, and no mortalities were recorded. Only one shrimp exposed to bacteria grown on marine agar showed some low grade AHPNS gross signs. The histological examination results of the other 2 treatments (TSA+ and sheep blood agar) were negative for AHPNS.

Regarding the treatments with the individual cultures A/1, A/2, and A/3 grown in liquid media (TSB+), the results clearly indicated that only one type of colony from the mixed culture could replicate the AHPNS pathology in experimental shrimp (Fig. 4D). Only shrimp in the treatment with bacterial isolate A/3 presented gross signs of AHPNS and mortalities. In contrast to this result, the *Penaeus vannamei* in the 2 other treatments (A/1 and A/2)

showed no signs of AHPNS by subsequent histological examination (Fig. 4B,C). Meanwhile, shrimp in the A/3 group were positive for AHPNS at high severity grades (Fig. 4D). Shrimp in the A/3 group approached 100% mortality on Day 2 of the challenge test; in contrast, no mortalities were recorded in either the A/1 or A/2 groups (Fig. 5, Table 1). These results indicated that in the mixed culture A, there is probably only one type of bacteria capable of inducing AHPNS pathology. The results also indicated that liquid media might be required for the suspected AHPNS-causing bacteria to be able to induce the disease.

Expt 3: Immersion bioassay with bacteria isolated from AHPNS-positive treatments of Expt 2

Expt 2 clearly indicated that only one bacterial isolate (A/3) from mixed culture A could induce AHPNS. From shrimp challenged with isolate A/3, we re-isolated the same type of colony from an experimentally infected shrimp's stomach and repeated the immersion experiment using the same method described above. Moreover, the same bacterial isolate was also recovered from the seawater from the positive control tank of the previous immersion study (immersion with mixed culture A). This recovered bacterial isolate was designated as B/1. In Expt 3, we tested both isolates (A/3 and B/1) recov-

Table 1. *Penaeus vannamei*. Summary of immersion Expts 1, 2, and 3. AHPNS: acute hepatopancreatic necrosis syndrome; TSA+ (TSB+): tryptic soy agar (broth) with added 2% sodium chloride. Samples considered positive for AHPNS were evaluated using the G-grading system, ranging from G0 for negative to G4 indicating the highest severity

Treatment	Media used	Number of shrimp	Cumulative mortality (%)	AHPNS histology
Expt 1				
Mixed culture A	TSB+	45	100	AHPNS positive G3–4
Negative control	Sterile TSB+	15	0	Negative
Expt 2				
Mixed culture A (positive control)	TSB+	15	100	AHPNS positive G3–4
Pure culture A/1	TSB+	15	0	Negative
Pure culture A/2	TSB+	15	0	Negative
Pure culture A/3	TSB+	15	100	AHPNS positive G3–4
Mixed culture A (TSA+)	TSA+	15	0	Negative
Mixed culture A (marine agar)	Marine agar	15	0	Suspected AHPNS
Mixed culture A (blood agar)	Sheep blood agar	15	0	Negative
Negative control	Sterile TSB+	15	0	Negative
Expt 3				
Mixed culture A (positive control)	TSB+	15	100	AHPNS positive G3–4
Pure culture A/3	TSB+	15	100	AHPNS positive G3–4
Pure culture B/1	TSB+	15	100	AHPNS positive G3–4
Reverse gavage A/3	Filtered TSB+	15	100	AHPNS positive G3–4
Negative control	Sterile TSB+	15	0	Negative

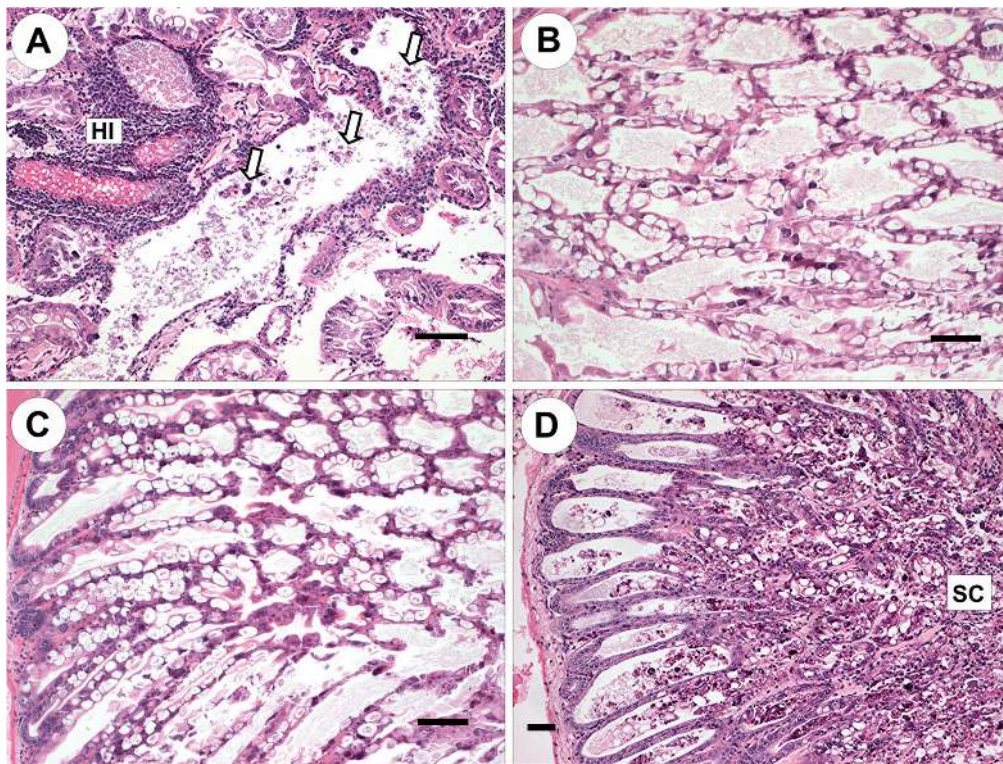


Fig. 4. *Penaeus vannamei*. Histological sections from the immersion bioassay showing the AHPNS lesions induced by pathogenic bacterial isolates. (A) Affected hepatopancreas (HP) of positive control (mixed culture A immersion), showing tubule epithelial cells sloughing (examples marked by arrows) and hemocytic infiltration (HI) surrounding remnants of necrotic HP tubules with sloughed HP cells in their lumens. (B,C) Normal HP tubule structure of A/1 and A/2 immersion. (D) Affected HP of shrimp in the A/3 immersion showing acute HP tubule degeneration and significant sloughing (SC) of HP tubule epithelial cells; the pathology progresses from proximal to distal. Scale bars = (A,B,C) 100 μ m, (D) 50 μ m

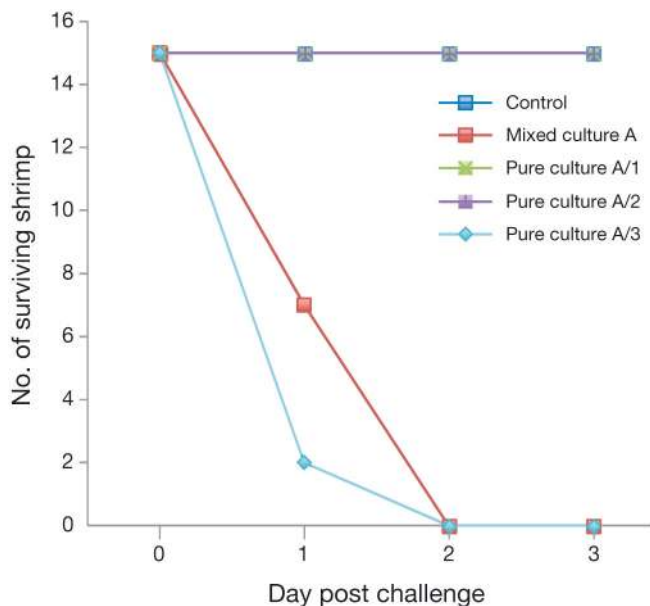


Fig. 5. *Penaeus vannamei*. Mortalities induced in the immersion bioassay using the mixed and pure bacterial cultures grown in liquid media

ered from Expt 2 for pathogenicity. The positive control group was treated with the known, consistently pathogenic, mixed bacterial culture A that originated in Vietnam. All isolates were grown in liquid media (TSB+) for the challenge study. One additional treatment was also designed to test for toxicity of liquid media that had been inoculated with A/3, but had the bacterial cells removed by filtration using 0.2 μ m filters. The reverse gavage technique (Aranguren et al. 2010) was applied to challenge SPF *Penaeus vannamei* in this treatment.

The results of this experiment confirmed that mixed culture A and A/3 caused AHPNS pathology both with typical AHPNS gross signs and histological changes (Fig. 6A,B). In addition, the recovered isolate B/1 from seawater of Expt 2 (which had an identical colony morphology as the A/3 isolate), also induced typical AHPNS pathology as was seen by both gross signs and histology (Fig. 6C). Interestingly, the reverse gavage treatment also caused AHPNS gross signs as well as histological changes at the microscopic level, characterized by acute HP cell

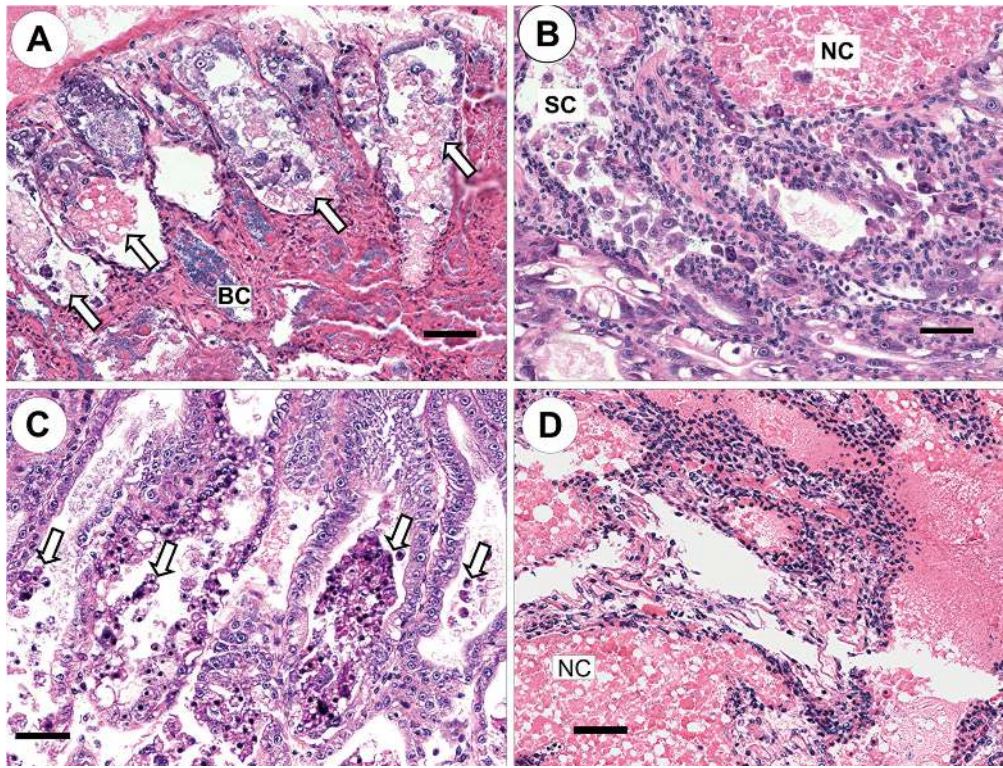


Fig. 6. *Penaeus vannamei*. Histological sections from the immersion and reverse gavage bioassays showing AHPNS lesions. (A) Mixed culture A immersion treatment. Arrows: examples of hepatopancreas (HP) tubule epithelial cells sloughing into the HP tubule lumens. Significant bacterial colonization (BC) can also be observed within remnants of HP tubules. (B) Pure culture A/3 immersion treatment: no B- or R-cells are apparent. HP tubule epithelial cells are shown sloughing (SC) into the HP tubule lumens. (C) Pure culture B/1 immersion treatment. No B- or R-cells are apparent. Arrows: examples of acute cell sloughing of HP tubule epithelial cells. (D) Reverse gavage treatment: shown are necrotic and sloughed HP tubule epithelial cells (NC) and marked hemocytic infiltration among HP tubules. Scale bars = 50 μ m

sloughing and inter- and intra-tubular hemocytic infiltration (Fig. 6D). Shrimp treated on 2 consecutive days by reverse gavage with 0.2 μ m-filtered supernatant from TSB+ (media was inoculated with A/3 and incubated for 18 h at 28°C) showed typical AHPNS pathology.

In general, experimental shrimp subjected to immersion treatment with pure cultures (A/1 and B/1) showed acute progression of AHPNS, characterized by rapid development of typical gross signs. Mortality in treatments with pure cultures of bacteria occurred after 18 h of exposure and approached 100% after 48 h of exposure. Mortality in the treatment with the mixed culture A was slower than with the treatments with pure cultures. Similarly, shrimp in the reverse gavage treatment tended to survive longer than those subjected to the bacterial immersion treatments (Fig. 7). However, the AHPNS histological lesions in reverse gavage treatments were very similar to those exhibited in shrimp from the treatments with immersion of bacterial suspensions

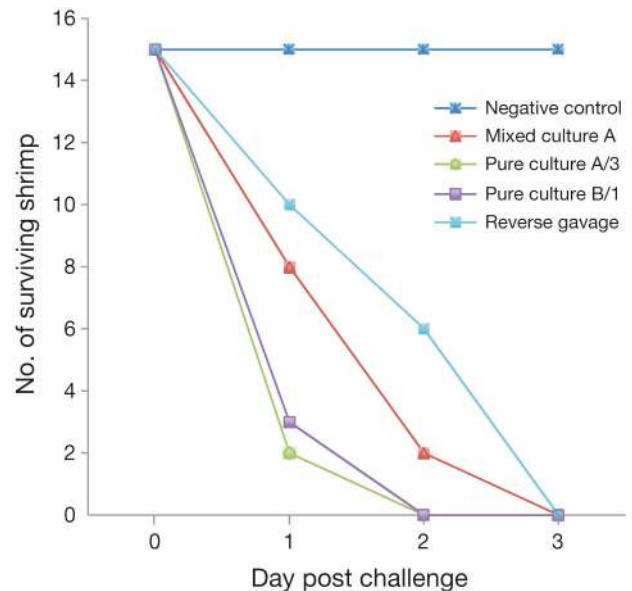


Fig. 7. *Penaeus vannamei*. Mortalities induced in the immersion and reverse gavage bioassays using the mixed and pure bacterial cultures grown in liquid media

(Fig. 6D). These results indicate that only one type of bacterial colony is responsible for causing AHPNS pathology in laboratory conditions, and that a filterable toxin is responsible for the observed pathology to the HP.

Bacterial identification

The only bacterial isolate (pure cultures A/3) from the mixed culture that showed pathogenicity in this study was identified as *Vibrio parahaemolyticus* using API Rapid NE test kits and 16S rRNA sequencing. The detailed taxonomy of the bacterial species that causes AHPNS will be reported in a subsequent paper (L. Nunan et al. unpubl.).

DISCUSSION

Based on the nature of AHPNS pathology, a series of infectivity studies was designed to identify the source and mode of infection. The on-site studies in Vietnam took advantage of the location in an AHPNS-affected region to conduct several studies using fresh, never frozen, samples of *Penaeus vannamei* and *P. monodon* exhibiting gross signs of AHPNS. Subsequent histological examination determined the positive AHPNS status for the inocula that were prepared from locally obtained AHPNS-positive shrimp. The on-site studies indicated that AHPNS could not be transmitted through intramuscular injection challenges, while *per os* and co-habitation studies confirmed that AHPNS was an infectious disease.

Histopathology studies indicated that in the early phase of the disease, the only affected organ is the HP. HP malfunction is characterized by tubule epithelial cells sloughing into the HP tubule lumens well before there is any indication of a causative agent (e.g. causative bacteria). This was the impetus for focusing on the stomachs of AHPNS-positive shrimp as a probable source of the causative agent or toxin of AHPNS, and efforts were made to culture aseptically excised stomachs from affected *Penaeus monodon*.

The initial infectivity studies conducted in Vietnam, using a mixture of bacteria isolated from AHPNS-positive stomachs and grown in liquid media, were found to produce AHPNS pathology in SPF shrimp via an immersion challenge test. This result was a strong indication that AHPNS is caused by a cultivable, infectious agent that could be found

in stomachs of infected shrimp. Furthermore, these studies using the bacterial agent of AHPNS satisfied the 4 points of Koch's Postulates (Lightner 1988, Saulnier et al. 2000, Hasson et al. 2009). The reasons why bacteria grown on solid media did not cause AHPNS were not determined. However, cell to cell signaling in broth culture, as opposed to lack of space for this activity among bacteria grown on solid media, may be an area for future studies.

The results from this research also indicated that the bacteria-free supernatant of the broth media could induce AHPNS pathology in the reverse gavage experiment. This evidence strongly suggests that AHPNS lesions are caused by bacterial toxin(s), as was suggested in a previous paper on AHPNS pathology (Lightner et al. 2012).

Future work will focus on characterizing the agent of AHPNS using traditional biochemical methods as well as 16S rRNA sequencing (L. Nunan et al. unpubl.), developing diagnostic methods (possibly using PCR to detect the toxin-producing gene of the agent), and developing methods that may be useful at managing AHPNS in affected countries.

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