

NOTE

Preservation of necrotizing hepatopancreatitis bacterium (NHPB) by freezing tissue collected from experimentally infected *Litopenaeus vannamei*

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ABSTRACT: Necrotizing hepatopancreatitis (NHP), a severe disease of penaeid shrimp, is caused by bacteria (NHPB) that have previously been demonstrated to reside in tubular epithelial hepatopancreatic (HP) cells of infected shrimp. There has yet to be a successful *in vitro* culture method to grow the intracellular organism; therefore, it must be propagated *in vivo* via transmission from NHPB-infected shrimp to healthy individuals. In our studies, NHPB propagation tanks containing infected shrimp were used to maintain a constant supply of organisms for experiments. In order to develop a method for storing infectious NHPB material for future challenge studies, we collected HP tissue containing NHPB by flash freezing whole, fresh HPs at -80°C for up to 80 d and used it to successfully infect specific pathogen-free *Litopenaeus vannamei* per os in controlled experiments. HP tissue samples were collected from dead shrimp, and PCR was performed to confirm the presence of NHPB. Our results demonstrate that the infectivity of NHPB in tissue is not altered after being frozen at -80°C when compared to NHPB in fresh tissue. Thus, the continual propagation of NHPB *in vivo* is not required to assure a source of the infectious agent.

KEY WORDS: Aquaculture · NHPB · *Litopenaeus vannamei* · Frozen tissue · Rickettsiae

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INTRODUCTION

Necrotizing hepatopancreatitis (NHP) is an economically important disease of cultured penaeid shrimp, including *Litopenaeus vannamei*, which has been responsible for significant losses in Texas shrimp farms (Frelier et al. 1992, Lightner 2000). Reports of disease outbreaks are often associated with elevated temperature (30 to 35°C) and salinity (30 to 38 ppt) of ponds, and the disease has resulted in mortality rates of 20 to 95% (Lightner & Redman 1994, Loy et al. 1996).

NHP is caused by a single, pleomorphic, gram-negative, intracellular bacteria (Frelier et al. 1992, Lightner et al. 1992). NHP bacteria (NHPB) are concentrated within tubular epithelial hepatopancreatic

(HP) cells of penaeid shrimp (Freiler et al. 1992). Successful attempts to culture NHPB *in vitro* are unreported; therefore, for experimental purposes it has been necessary to propagate the organism in live shrimp. This process is expensive and time-consuming as susceptible shrimp must continuously be added to NHPB-infected tanks to reproduce the disease.

It was recently demonstrated that individual *Litopenaeus vannamei* can be infected with NHPB by per os exposure to fresh, NHPB-infected tissue (Vincent et al. 2004). Due to the difficulty of collecting large amounts of infective tissue, experimental studies involving an extensive number of shrimp can be problematic to conduct. Therefore, a method for preservation of NHPB *in vitro* would facilitate experimentation

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by allowing for collection of material over an extended period of time.

The purpose of this study was to evaluate the infectivity of NHPB in tissue frozen at -80°C and to develop a model for infection of *Litopenaeus vannamei* with NHPB that would eliminate the need for long term *in vivo* propagation of the organism. We demonstrate that the infectivity of NHPB is unaffected by freezing at -80°C .

MATERIALS AND METHODS

Post larvae specific pathogen free (SPF) shrimp.

SPF *Litopenaeus vannamei* were obtained from the Oceanic Institute, Kailua-Kona, Hawaii, USA. SPF shrimp are certified negative for yellow head virus, Taura syndrome virus, white spot syndrome virus, infectious hypodermal and hematopoietic necrosis virus, *Baculovirus penaei*, *Monodon baculovirus*, hepatopancreatic parvovirus, infectious myonecrosis virus, *Microsporidians microsporidia*, *Haplosporidians haplosporidia*, *Gregarines apicomplexa*, and NHPB. *Vibrio* spp. were found to be present as normal flora in SPF shrimp (B. H. Janke, Iowa Veterinary Diagnostic Laboratory, Ames, IA, unpubl. data). Upon arrival, shrimp were placed in approximately 1360 l salt water tanks made with dechlorinated city water and Crystal Sea Marinemix, temperature $25 \pm 2^{\circ}\text{C}$, salinity 22 ± 2 ppt, with constant aeration (Marine Enterprises International). Each tank was equipped with a carbon filter (Proline activated charcoal, Aquatic EcoSystems) and a 4.73 l biofilter containing approximately 1.2 to 1.4 kg of crushed oyster shell. Initially, shrimp were fed commercial shrimp protein powder (approximately 5% shrimp body weight) twice daily (Shrimp Production formula 45/10, Rangen). As they grew, shrimp were placed accordingly into other tanks and fed commercial shrimp protein granules (approximately 5% shrimp bodyweight) twice daily (Rangen). Juvenile shrimp weighing 4 to 6 g were fed once d^{-1} to maintain their weight.

NHPB propagation tanks. An isolate of NHPB that originated in Texas, USA, was obtained from Amanda Vincent, Gulf Coast Research Laboratory, University of Southern Mississippi, Ocean Springs, Mississippi, USA. Approximately 15 NHPB-infected, whole, fresh shrimp carcasses were sent on ice from Mississippi and placed in a tank containing 15 live SPF shrimp. All shrimp were transferred to 606 l propagation tanks containing approximately 303 l of artificial salt water, temperature $30 \pm 2^{\circ}\text{C}$, salinity 30 ± 2 ppt. A total of 40 shrimp were kept in each tank. Tanks were equipped with a carbon filter, biofilter and constant aeration.

As shrimp in the propagation tanks died, their carcasses were left in the tanks and SPF shrimp were added to replace them. Shrimp were taken from these tanks and used as infectious material for all trials. Prior to the start of the 2 trials, NHPB lesions were confirmed in shrimp from the NHPB propagation tanks by histopathology using microscopy and immunohistochemistry staining (B. H. Janke unpubl.). Weekly, HP tissue samples from shrimp found dead in propagation tanks were tested by PCR to confirm the presence of NHPB. Over a 6 mo period, 202 HP tissue samples from the NHPB propagation tanks were examined by PCR and 99% tested positive for NHPB.

Trial 1. Preparation of NHPB inoculum: On Day 0, 6 SPF and 9 NHPB-infected shrimp were collected and placed in individual sterile whirl-pak bags and placed in a freezer at -80°C . After 3 min, shrimp were removed from the freezer, and each HP was aseptically removed, cut into several pieces, and placed into sterile, individual 1.5 ml Eppendorf tubes without diluent. Three of 6 SPF HPs and 6 of 9 NHPB-infected HPs were held in a -80°C freezer for either 1 or 5 d prior to their use as inoculum. Three remaining SPF HPs and 3 NHPB-infected HPs were immediately placed into their respective experimental tanks.

Trial 1. Experimental tanks: Ten SPF shrimp ranging in size from 4 to 6 g were placed into each of five 227 l tanks containing approximately 133 l of artificial salt water, temperature $30 \pm 2^{\circ}\text{C}$, salinity 30 ± 2 ppt. Tank setup and feeding were as described above. Shrimp were held in tanks for several weeks until the start of the study. On Day 0 of Trial 1, 3 fresh, unfrozen SPF HPs and 3 NHPB-infected HPs were placed into each of 2 tanks. On Day 1, 3 SPF HPs and 3 NHPB-infected HPs were removed from the -80°C freezer and placed into each of 2 other tanks. On Day 5, 3 NHPB-infected HPs were removed from the -80°C freezer and placed into the fifth tank. Shrimp were fed protein granules (approximately 5% shrimp body weight) once daily (Rangen). Temperature and salinity were monitored once daily and fresh water was added only to replace water loss from evaporation. Nitrite levels were monitored weekly using a nitrite test kit (Hach Chemical). Each day tanks were observed for dead shrimp, which were immediately removed and placed into sterile whirl-pak bags. Date of death was recorded, the HP was aseptically removed, and a 25 mg sample was taken for PCR.

Trial 2. Preparation of NHP inoculum: Eighty days prior the start of the study, 4 shrimp from the NHPB propagation tanks were collected and each HP was obtained, as in Trial 1, and placed in a -80°C freezer until the start of the trial. Approximately 25 mg of each HP was taken for PCR before freezing. Four shrimp from NHPB propagation tanks were collected

and processed similarly 50 and 5 d prior to the start of the study. Four SPF shrimp were also collected and processed 50 d prior the start of the study. On Day 0, 4 SPF shrimp and 4 shrimp from NHPB propagation tanks were collected and processed as above, but their HPs were not placed into the freezer. They were held at 4°C until all were collected, and then quickly placed into experimental tanks. HPs were each cut into 3 to 4 pieces before being placed into experimental tanks.

Trial 2. Experimental tanks: A total of 6 tanks were used in Trial 2. Ten SPF shrimp ranging in size from 4 to 6 g were placed into each of the six 227 l tanks, set up as in Trial 1. These SPF shrimp were held in the 6 tanks for several weeks until the start of the study. On Day 0 of Trial 2, HPs collected 80 d (4 HPs), 50 d (8 HPs), and 5 d (4 HPs) prior to the start of the study, as well as on Day 0 (8 HPs), were removed from the freezer. Four HPs from each of the experimental groups (Table 1) were placed into each of 6 tanks. Shrimp were then observed for 15 min, to ensure that the HPs were being eaten. Shrimp were fed protein granules (approximately 5% shrimp body weight) once daily (Rangen). Temperature, salinity and nitrite levels were monitored as in Trial 1. Shrimp found dead in tanks were processed as in Trial 1.

PCR. Extraction procedure: Total genomic DNA was extracted from each HP sample according to the protocol for 'Isolation of nucleic acids from mammalian tissue' found in the High Pure PCR Template Preparation Kit (Roche Applied Science). After the addition of tissue lysis buffer and Proteinase K, samples were placed

in a 55°C water bath overnight for complete lysis. Each DNA sample was resuspended in 100 µl of elution buffer rather than 200 µl in order to increase sample DNA concentration. DNA was kept at 4°C to be tested by PCR within 48 h and stored at -20°C for long-term use.

PCR setup and run: NHPB primer sequences, positive control DNA and PCR protocol were obtained from Dr. Donald Lightner, University of Arizona, Tucson. NHPB PCR was conducted using puReTaq Ready-To-Go PCR Beads (Amersham Biosciences). Primers used were NHPf2: 5'- CGT TGG AGG TTC GTC CTT CAG T - 3' and NHPr2: 5'- GCC ATG AGG ACC TGA CAT CAT C - 3'. PCR reactions were performed in a GeneAmp PCR System 9700 (PE Applied Biosystems). Cycling conditions were as follows: Step 1: 95°C for 2 min, 1 cycle; Step 2: 60°C for 30 s, 72°C for 30 s, 95°C for 30 s, 25 cycles; Step 3: 60°C for 1 min, 72°C for 2 min, 1 cycle; 4°C infinite hold. PCR products were run on a pre-cast 2% Invitrogen E-gel (Invitrogen) in order to confirm desired product size via comparison to a pGEM DNA ladder (Promega). DNA was visualized using GelDock (Bio-Rad Laboratories).

RESULTS

Two separate trials were conducted in which juvenile SPF *Litopenaeus vannamei* were infected with NHPB by per os exposure to *L. vannamei* HP tissue, either fresh or previously frozen at -80°C for 80, 50, 5, or 1 d. Survival analysis was performed for each trial

Table 1. *Litopenaeus vannamei*. Per os exposure of specific pathogen free *L. vannamei* to frozen hepatopancreatic material infected with necrotizing hepatopancreatitis bacterium (NHPB). PCS: positive control shrimp fed fresh, NHPB-infected hepatopancreas; E1: shrimp fed NHPB-infected hepatopancreas (HP) tissue frozen at -80°C for 24 h; E5: shrimp fed NHPB-infected HP frozen at -80°C for 5 d; E50: shrimp fed NHPB-infected HP frozen at -80°C for 50 d; E80: shrimp fed NHPB-infected HP frozen at -80°C for 80 d; NCS: negative control shrimp fed fresh, uninfected HP; NCZ: negative control shrimp fed uninfected HP frozen at -80°C for 1 d (Trial 1) or 50 d (Trial 2). In Trial 1, group PCS shrimp were terminated 58 d post exposure to infective NHPB material; group E1, 57 d post exposure; and group E5, 53 d post exposure. All groups within Trial 2 were terminated 60 d post exposure. CL: confidence limits

	Total no. of shrimp tank ⁻¹	No. of shrimp exposed to NHPB	% survival on day of trial termination	% NHPB positive by PCR by day of trial termination	Median survival time (d) (95% CL)
Trial 1					
PCS	10	10	0	80	40 (21-42)
E1	10	10	10	80	49 (21-49)
E5	10	10	10	100	43 (22-47)
NCS	10	0	100	0	-
NCZ	10	0	100	0	-
Trial 2					
PCS	10	10	30	50	47 (28-60+)
E5	10	10	0	70	28 (10-32)
E50	10	10	10	80	30 (15-53)
E80	10	10	10	80	21 (15-55)
NCS	10	0	80	0	-
NCZ	10	0	90	0	-

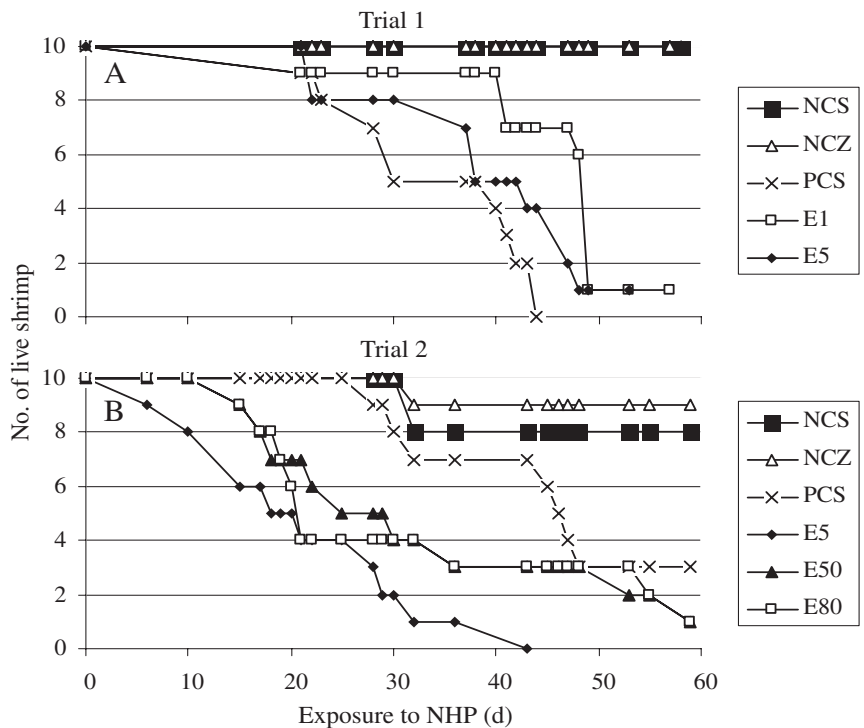


Fig. 1. Ten shrimp per experimental group were fed either fresh or frozen (-80°C) hepatopancreas (HP) from necrotizing hepatopancreatitis bacteria (NHPB)-infected and specific pathogen free shrimp. Each group was housed in a separate tank. (A) Survival curves for shrimp in Trial 1. (B) Survival curves for shrimp in Trial 2. NCS: negative control shrimp fed fresh, uninfected HP. NCZ: negative control shrimp fed uninfected HP frozen at -80°C for 1 d (Trial 1) or 50 d (Trial 2). PCS: positive control shrimp fed fresh, NHPB-infected HP. E1: shrimp fed NHPB-infected HP frozen at -80°C for 24 h. E5: shrimp fed NHPB-infected HP frozen at -80°C for 5 d. E50: shrimp fed NHPB-infected HP frozen at -80°C for 50 d. E80: shrimp fed NHPB-infected HP frozen at -80°C for 80 d

and median survival time was calculated for each tank using JMP 5.1.2 (SAS Institute) (see Table 1). Data was censored for any shrimp whose carcass was not found in the tank either due to cannibalism or unknown reasons, and for any shrimp that survived to trial termination. It was determined that frozen HP material from shrimp previously infected with NHPB successfully infected SPF *L. vannamei*.

In Trial 1, 10 of 10 shrimp in each of 2 negative control tanks (NCS and NCZ) survived until termination of the study (see Fig. 1). At the termination of the study, all 20 negative control shrimp tested negative for the presence of NHPB by PCR. In the positive control tank (PCS), all 10 shrimp were found dead prior to the termination of the study. At the termination of the study, 8 of 10 shrimp in the PCS tested positive for the presence of NHPB by PCR. In the experimental tank fed NHPB-infected shrimp frozen at -80°C for 5 d (E5), 9 of 10 shrimp were found dead prior to the termination of the study. At the termination of the study, 10 of 10 shrimp in this tank tested positive for the presence of NHPB by

PCR. In the experimental tank fed NHPB-infected shrimp frozen at -80°C for 1 d (E1), 9 of 10 shrimp were found dead prior to the termination of the study. At the termination of the study, 8 of 10 shrimp in this tank tested positive for the presence of NHPB by PCR.

In Trial 2, an error occurred on Day 32 in which all 6 tanks did not have aeration for approximately 18 h, leading to the death of shrimp in several tanks, including 3 SPF shrimp from NCS and NCZ (see Fig. 1). Eight of 10, and 9 of 10 shrimp from each of NCS and NCZ survived until the termination of the study. At the termination of the study, 20 of 20 shrimp from NCS and NCZ tested negative for NHPB by PCR. In PCS, 7 of 10 shrimp were found dead prior to the termination of the study. At the termination of the study, 5 of 10 shrimp in PCS tested positive for the presence of NHPB by PCR. In the experimental tank fed NHPB-infected shrimp frozen at -80°C for 80 d (E80), 9 of 10 shrimp were found dead prior to the termination of the study. At the termination of the study, 8 of 10 shrimp in this tank tested positive for the presence of NHPB by PCR. In the experimental tank fed NHPB infected shrimp frozen at -80°C for 50 d (E50), 9 of 10 shrimp were found dead prior to the termination of the study. At the termination of the study, 8 of 10 shrimp in this tank tested positive for the presence of NHPB by PCR. In the experimental tank fed NHPB infected shrimp frozen at -80°C for 5 d (E5), 10 of 10 shrimp were found dead prior to the termination of the study. At the termination of the study, 7 of 10 shrimp in this tank tested positive for the presence of NHPB by PCR.

For both Trials 1 and 2, the actual amount of organism within each HP fed to experimental shrimp was not known. For Trial 2, a 25 mg piece of each HP to be used as inoculum was taken for PCR before the rest of each HP was placed in each tank. In Trial 2, only 1 of 4 HPs placed in the positive control tank fed fresh material (PCS) tested positive for NHPB by PCR, whereas 4 of 4 HPs administered to each tank (E5, E50 and E80) given frozen, infective material tested positive by PCR. Therefore, although the exact difference in dosage between each tank was not known, it is known that in Trial 2 the PCS tank received only 1 PCR-positive HP while each of the other tanks received 4.

DISCUSSION

The purpose of this research was to develop a method of collecting and storing NHPB-infected material for an extended time period to allow for a more controlled procedure for transmitting NHPB to susceptible *Litopenaeus vannamei*. Our method involved collecting and freezing HPs at -80°C from shrimp infected *in vivo* with NHPB, and using this HP material to infect SPF shrimp with NHPB by per os exposure to frozen material. One advantage of this NHPB preservation method is that tissue can be stored until there is enough for multiple experimental exposures.

One of the difficulties encountered when conducting this study was attempting to standardize the inoculum given to each tank. For Trial 1, PCR was not performed on HPs used as inoculum, but in Trial 2, PCR samples were taken from the HP inoculants to determine if NHP bacteria were present in each tank at the start of the trial. However, the PCR protocol did not allow for quantification of inoculum; therefore, variation in survival rates (Table 1) may be due to dosage administered to each tank.

For both Trials 1 and 2, all of the shrimp that were dead before the termination of each study in the PCS and experimental tanks did not test positive for NHPB by PCR due in part to the cannibalistic nature of the shrimp. Some shrimp found dead in tanks were almost completely consumed by other shrimp in the tank; therefore, there was no hepatopancreas material to test for NHPB by PCR.

Previous challenge studies with *Vibrio cholerae* have demonstrated that inoculum prepared directly from frozen organism without further incubation was able to cause illness typical to that of cholera (Sack et al. 1998, Cohen et al. 1999). An advantage of using frozen inoculum is that multiple studies may be conducted over an extended period of time using the same passage number for each study.

A limited number of studies on NHPB have been conducted with just a few isolates or in isolates procured from the wild but not preserved. The preservation method reported here should allow for future studies on multiple isolates, assuming other isolates of NHPB can be preserved in a similar manner. One reason for the success of this study may be due to the method used to prepare the HPs prior to inoculation. Shrimp collected for use as inoculum were removed from the tank and immediately placed in a sterile whirl-pak bag, and held at -80°C for 3 min, and then their HPs were quickly removed and placed back into the -80°C freezer, or used directly as inoculum. Frelier et al. (1993) reported that sucrose gradient purified preparations of NHPB could be preserved by freezing; however, this approach was not evaluated in our studies.

The range of survival for *Litopenaeus vannamei* infected with NHPB by per os exposure as reported by Vincent et al. (2004) is 16 to 51 d with a median survival time of 34.5 d. Our study demonstrated similar findings with a median range of survival for NHPB-infected *L. vannamei* of 40 to 49 d for Trial 1 and 21 to 47 d for Trial 2. Differences in median range between these 2 trials may be due primarily to differences in inoculum given to each tank, as we were unable to quantify the amount of organism given to each tank. These results show that freezing NHPB in tissue does not affect its viability. The work done by Vincent et al. (2004) involved the infection of individual SPF *L. vannamei* with NHPB per os to fresh HP tissue, whereas the work described here involved the infection of SPF *L. vannamei* with either fresh or frozen HP tissue.

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