

6.2.1 Necrotizing Hepatopancreatitis of Penaeid Shrimp

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A. Name of Disease and Etiological Agent

Necrotizing Hepatopancreatitis (NHP), initially referred to as Texas Pond Mortality Syndrome (McVey, 1993), was first reported as a seasonal disease problem in Texas pond-reared *Litopenaeus vannamei* (Pacific white shrimp) populations (Johnson, 1990; Frelier et al., 1992). The etiologic agent was identified as a dimorphic, obligate intracellular rickettsial-like organism (RLO) that targets the shrimp hepatopancreas (Frelier et al., 1992; Lightner et al., 1992) and was classified as a member of the α -Proteobacteria group (Loy et al., 1996a). Ultrastructural studies have demonstrated the presence of a predominant rod form (0.25 X 0.9 μ m), as well as a helical form (0.25 X 3.5 μ m) with 8 flagella at its basal apex. Since this organism is non-culturable in *in vitro* systems, its life cycle has not been fully elucidated and speculated to be vector-mediated (Frelier et al., 1993; Loy et al., 1996a).

B. Known Geographic and Host Ranges of the Diseases

1. Geographical range.

Since its initial detection in 1985, NHP has been reported in various countries in the Western Hemisphere, including the United States, Belize, Guatemala, Columbia, Ecuador, Nicaragua, Costa Rica, Brazil, Peru, Mexico, Panama and Venezuela. (Jory, 1997; Lightner, 1996; Bradley-Dunlop et al., 2004)

2. Host Species.

Natural NHP infections have been detected in *Litopenaeus vannamei*, *L. setiferus*, *L. stylirostris*, *Farfantepenaeus aztecus* and *F. californiensis* (Lightner, 1996).

C. Epizootiology

Since mortality rates can vary between 20-95%, economic losses can be significant to commercial shrimp producers (Loy et al., 1996b). NHP infections appear to have a seasonal occurrence in both cultured and wild shrimp populations with natural clinical outbreaks associated with increases in both water temperature and salinity (Lightner & Redman, 1994; Frelier et al., 1995). This association with these two environmental factors may reflect an

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increase in pathogen virulence and/or vector mediation. In a recent experimental *per os* study, salinity was demonstrated to not be a major determining factor in the transmission of the NHP organism (Vincent & Lotz, 2007).

Transmission is horizontal via ingestion. Cannibalism is believed to be a major contributing factor to the rapid spread of infection within a shrimp pond population (Vincent et al., 2004). In endemic regions, routine monitoring of ponds is recommended when existing environmental conditions are conducive for clinical disease (Bondad-Rantaso et al., 2001). Early detection has been demonstrated to be important for successful control with oxytetracycline-medicated feeds (McVey, 1993; Brock & Main, 1994; Lightner & Redman, 1994).

D. Disease signs

The clinical signs displayed by infected shrimp are nonspecific in nature and characterized by lethargy, reduced feed intake, decreased growth rate, softened shell and an atrophied hepatopancreas (Brock & Main., 1994; Lightner, 1996; Bondad-Reastasco et al., 2001). Variable pond mortality rates of up to 95% have been reported. Infected shrimp display empty midguts on pond-side exam.

The hepatopancreas is the target tissue for this organism. Infected cells initially become hypertrophied due to the intracytoplasmic proliferation of this organism. Three stages of infection were defined by Frelier et al. (1992). In a *per os* time course study, early NHP infection (stage I) was detected in the resorptive, fibrillar and/or B cells of infrequent scattered tubules by 6 days post exposure (Vincent & Lotz, 2005). Stage II of NHP infection was demonstrated to occur at 16-37 d post-exposure with moderately frequent scattered to widespread tubular epithelial cell hypertrophy/attenuation present and associated with variable numbers of intracytoplasmic rickettsial-like organisms. Stage III was characterized by frequent scattered tubular necrosis and mortality at 16 to 51 days post-exposure.

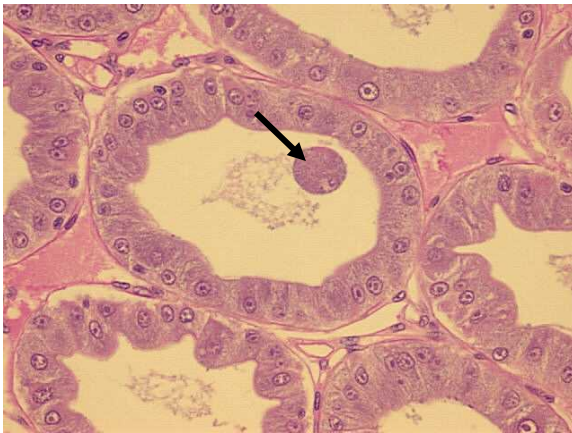


Figure 1. The generalized basophilic granularity to the cytoplasm of the tubular epithelial cells is due to the presence of numerous intracytoplasmic rickettsial-like organisms. A desquamated rickettsial-infected cell (arrow) is present within a tubule (H&E; 400X).

E. Disease Diagnostic Procedures

Presumptive

1. Gross observations

Affected shrimp may display a variety of gross and/or clinical signs that can be used to identify suspect infected shrimp to submit for diagnostic testing (Brock & Main, 1994; Lightner, 1996; Vincent et al., 2004). These may include decrease in the size of the hepatopancreas, empty midgut (Figure 2), reduced growth, soft flaccid shells and/or increased surface fouling with epibiotic/opportunistic organisms (e.g. black spot disease, etc.).

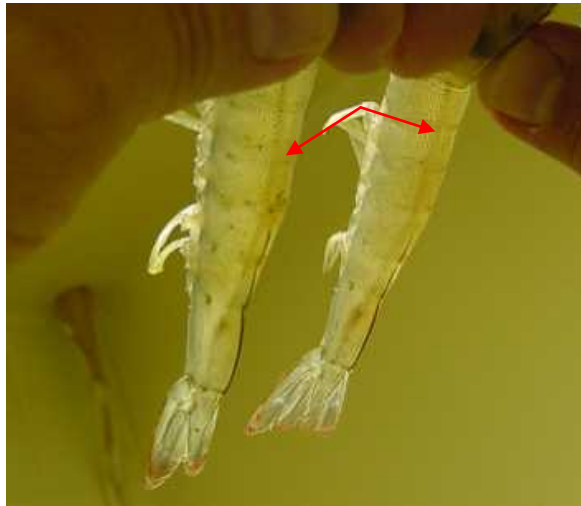


Figure 2. Gross examination for midgut fullness. Both midguts are empty in this view (arrows). The midgut will appear much darker (almost black) when ingesta is present.

2. Wet Mount Analysis

Squash preps of the hepatopancreas from suspect shrimp (Figure 3) can be evaluated microscopically for the presence of melanized necrotic tubules and/or reduced lipid content (Lightner, 1996). The presence of necrotic tubules, however, can also be due to infection by extracellular bacteria.

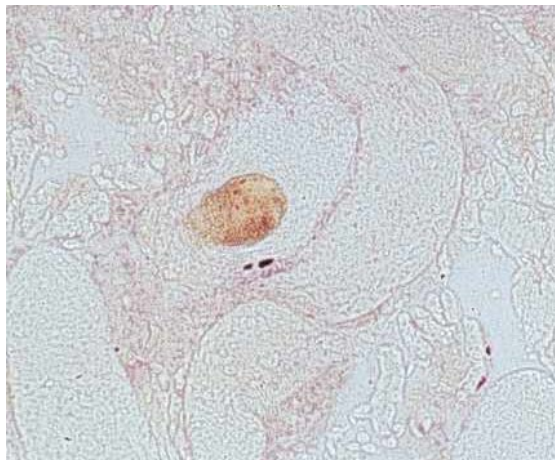


Figure 3. Necrotic tubule in squash prep made of the hepatopancreas (200X).

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Histopathology

Davidson-fixed shrimp tissues (Humason 1972) are processed, sectioned and stained with a modified Mayer's hematoxylin and Phloxine/eosin stain (H&E) as described by Bell & Lightner (1988). In NHP-infected shrimp, varying degrees of epithelial hypertrophy/attenuation, lipid reduction and tubular necrosis may be detected in microscopic examination of H&E-stained histologic sections of the hepatopancreas (Frelief et al., 1992; Lightner & Redman, 1994; Lightner, 1996). Infected hepatopancreocytes will have a generalized basophilic granularity to the cytoplasm (Figure 1). The presence of desquamated RLO-infected cells within the tubules is a common histologic finding in Stage II NHP infection. Other histologic features include interstitial hemocytic infiltrates and tubular dilatation. Steiner's & Steiner's stain (Frelief et al., 1992; Lightner, 1996) is a good special stain to demonstrate the presence of the intracytoplasmic NHP organisms as dark brown to black organisms within infected cells (Figure 4).

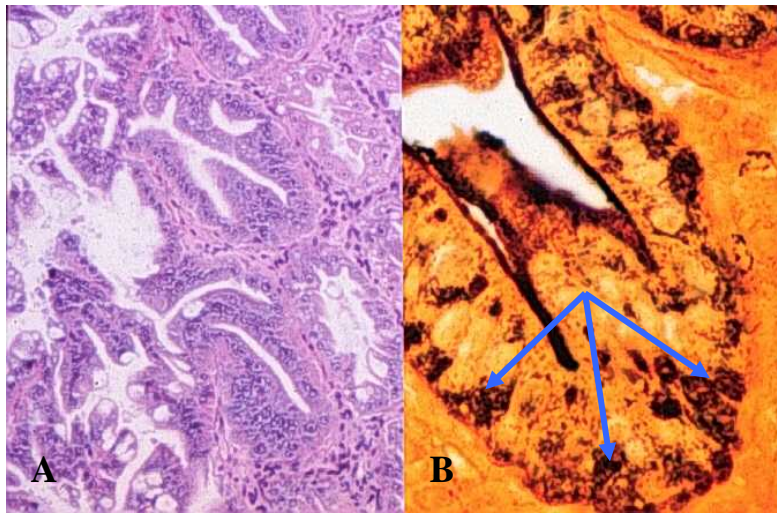


Figure 4. Stage II NHP-infected hepatopancreas with intracytoplasmic NHP organisms demonstrated with H&E (A; 200X) and Steiner's & Steiner's stain (B; 400X; arrows).

Confirmatory

1. *In situ* hybridization assay

In situ hybridization, using a PCR-generated, NHP-specific probe as outlined by Loy et al. (1996b), is performed to confirm suspect histologic findings. The NHP probe is generated by PCR and randomly-labeled with digoxigenin (see the following PCR reaction).

a. Slide processing

- i. De-paraffinize embedded tissue sections mounted on positively-charged slides in a xylene-based solvent.
- ii. Rehydrate tissue sections through a series of graded ethanols from 100% to 50% ethanol including one 10 min acid/ethanol soak.

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b. Permeabilization

- i. Incubate sections in Proteinase K for 8 min. at 37°C to permeabilize the tissues to allow probe penetration.
- ii. Inactivate the Proteinase K by heating slides up to 95-100°C for 10 min.
- iii. Then incubate the slide in 0.4% paraformaldehyde/PBS for 10 min at 4°C to enhance tissue adherence to the slide.

c. ISH hybridization & stringency washes

- i. Incubate tissue section(s) in the hybridization solution at 100°C for 10 min followed by a 2 hr (or overnight) incubation at 42°C.
- ii. Wash slides in decreasing concentrations of SSC (2X to 0.2X with the last soak at 37°C).

d. ISH detection step

- i. Incubate slides in an Anti-digoxigenin-alkaline phosphatase conjugate for 30 min at 37°C.
- ii. Then incubate in development solution (NBT/BCIP substrate) for 30-60 min. at 37°C.
- iii. Counterstain with Bismarck Brown stain and dehydrate slides through graded ethanols (95%-100%) and a xylene-based solvent prior to cover-slipping for histologic evaluation (Figure 5).

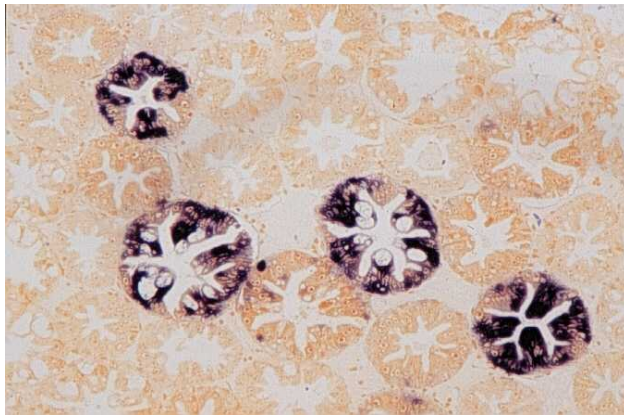


Figure 5. In situ hybridization of NHP-infected hepatopancreatic section with intracytoplasmic probe uptake (dark blue-black) by NHP-infected cells. Counterstain with Bismarck Brown stain (200X).

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2. Polymerase Chain Reaction (PCR)

NHP-specific PCR can be conducted on feces and/or hepatopancreata from suspect NHP-infected shrimp (Loy et al., 1996b; Brinez et al., 2003). Using NHP-specific oligonucleotide primers, a unique 209 bp target sequence, located within the 16S ribosomal RNA sequence of the NHP genome, is amplified exponentially by the polymerase chain reaction (Loy & Frelier, 1996c). The PCR-generated, NHP-specific amplicon is visualized via electrophoresis and UV-transillumination.

a. Sample processing

Genomic DNA is extracted and purified based on a protocol outlined by Loy et al (1996). A homogenate, made of 25-300 mg ethanol-preserved or fresh frozen hepatopancreatic tissue (or feces), is combined with lysis buffer and Proteinase K and incubated for 1-2 hr at 60-65°C. The Proteinase K is inactivated in a ten minute 95-100°C water bath. After centrifugation, 100 µl of the supernatant is purified on a spin column prior to use in the PCR assay.

b. DNA amplification

- i. Primers¹: NHP-F27: 5' ACA TGC AAG TCG AAC GCA ATA GG 3'
NHP-R235: 5' ACA GAT CAT AGG CTT GGT AGG CTG 3'
- ii. Master mix¹ (for 25 µl volumes):

Reagent	VolumeInitial	Concentration	FinalConcentration
10X Buffer	2.5µl	10X (15mM Mg)	1X (1.5mM Mg)
10X dNTPs	2.5µl	2.0 mM ea.	0.2 mM ea.
NHP-F 27	0.3µl	67.32µM	0.81µM
NHP-R235	0.3µl	67.32µM	0.81µM
Taq	0.25µl	5U/µl	1.25U/25µl rxn
H ₂ O	16.65µl	----	----
DNA	2.5µl	----	----

- iii. Cycling parameters¹:

Initial denaturation is conducted at 95°C for 5 min., followed by 35 cycles of:
45 sec at 95°C
45 sec at 58°C
45 sec at 72°C

With a final extension step of 10 min at 72°C.

1: Alternate primer set developed at University of Arizona (Lightner, unpublished data); generate 379 bp amplicon:

Forward (F2): 5' CGT TGG AGG TTC GTC CTT CAG T 3' (0.31 µM).

Reverse (R2): 5' GCC ATG AGG ACC TGA CAT CATC 3' (0.31 µM).

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Final concentration of the PCR master mix is similar to above, except for $MgCl_2$ at 2mM and AmpliTaq Gold at 2.5 units. Cycling parameters consist of 25 cycles each comprised of 30 sec intervals at 60°C, 72°C & 95°C with final extensions at 60°C for 1 min and at 72°C for 2 min.

c. Visualization of PCR products.

The PCR products are electrophoresed on a 2% agarose gel, stained with ethidium bromide and visualized with UV-transillumination. The generated banding pattern (209 bp amplicon) is compared to a reference DNA ladder and the gel photographed for documentation.

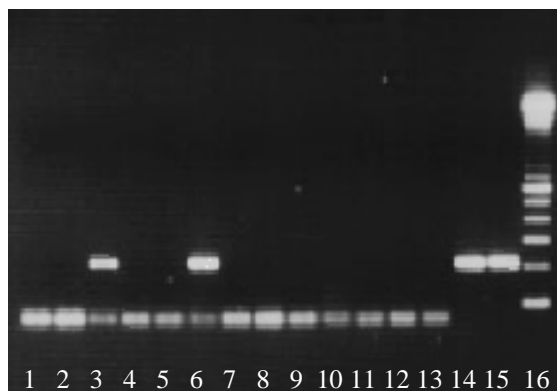


Figure 6. Ethidium bromide-stained gel demonstrating the 209 bp amplicon for NHP positive test samples (lanes 3 & 6), as well as for NHP positive extraction and molecular controls (lanes 14 & 15). Lane 1 is the negative control and lane 16 the reference DNA ladder.

3. Serology (e.g. dot-blot, immunohistochemistry)

Preliminary experimental investigations have demonstrated the feasibility for development of confirmatory serologic tests using monoclonal antibodies produced against the NHP bacterium (Bradley-Dunlop et al., 2004, Vincent & Lotz, 2005), but diagnostic reagents or kits are not commercially available at present.

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Table 1. Comparative table of diagnostic methods used for screening, presumptive diagnosis and confirmatory diagnosis of NHP infection.

Method	Relative sensitivity¹	Presumptive diagnosis²	Confirmatory Diagnosis
Gross signs	+	-	-
Histopathology	2-3	++	++
Serologic assays	3	+	+
PCR assays	3-4	+++	+++
ISH assays	4	+++	+++

¹0-4 increasing sensitivity

²applicability: - unsuitable, + limited utility, ++ standard method, +++ recommended method

F. Procedures for Detecting Subclinical Infections

1. Pond-side examinations of statistically significant numbers of shrimp should be conducted during times when environmental conditions are conducive for clinical disease expression. Selected samples should be injected with the appropriate fixative (Bell & Lightner, 1988):

- a. Davidson's fixative (Histology /in situ hybridization).
- b. Ethanol (PCR)

G. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability or Preservation of the Etiological Agent

1. Histologic/ in situ hybridization analysis

- a. Ship or transport triple-sealed, Davidson-fixed shrimp samples (injected whole body or hepatopancreata) in either Davidson's AFA fixative or 70% ethanol to an aquatic diagnostic laboratory for further diagnostic processing.

2. PCR analysis

- a. Ship or transport triple-sealed, ethanol-fixed shrimp samples (injected whole body or hepatopancreata) in 70-95% ethanol to an aquatic diagnostic laboratory for further diagnostic processing. Samples should be properly labeled (e.g. pond number, date, species, etc), and collected in a manner to minimize sample cross contamination, as well as maintained at or below 21 °C.

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