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NOTE

Morphological evidence for a single bacterial etiology in Texas necrotizing hepatopancreatitis in *Penaeus vannamei* (Crustacea: Decapoda)

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ABSTRACT: Texas necrotizing hepatopancreatitis (TNHP) is an economically significant disease of the marine shrimp *Penaeus vannamei* cultured in Texas, USA, shrimp farms. Since first recognized in 1985, the disease has occurred seasonally, and it has inflicted serious crop losses of 20 to 90% nearly every year in farms located in southern Texas. Two descriptive papers have been published on the histopathology and ultrastructure of the hepatopancreas of shrimp with TNHP, and the authors of both papers concluded that at least 2 distinctly different species of intracellular bacteria were present in the infected cells. Morphological evidence is presented in this paper that supports the contention that the 2 morphological types, a rickettsia-like form and a helical form as previously reported, are morphological variants of the same species of bacteria.

Since first encountered in 1985 (Johnson 1990, Frelier et al. 1992), Texas necrotizing hepatopancreatitis (TNHP) has come to be recognized as an economically significant disease of the marine shrimp *Penaeus vannamei* (Crustacea: Decapoda) cultured in Texas shrimp farms. The disease has occurred seasonally in most of the shrimp farms in the affected region of Texas, and it has inflicted serious crop losses (20 to 90%) nearly every year (Frelier et al. 1992, Lightner in press). Despite the distribution of 'seed stock' produced from stock reared in the affected area of Texas to other regions in the USA, Mexico, and Central America, TNHP has not been observed to date in *P. vannamei* or other *Penaeus* spp. reared outside of the affected region in Texas (Lightner in press).

Two descriptive papers have been published on the ultrastructure of the hepatopancreas of shrimp with TNHP. In the original paper in which the presence of intracellular bacteria was reported in TNHP, Krol et al. (1991) described 3 forms of microorganisms: (1) a pleomorphic rod-shaped rickettsia-like bacterium, (2) a helical form of a mollicute-like bacterium, and (3) a filamentous mollicute-bacterium. The rod-shaped rickettsia measured 300 nm wide by 900 nm long, was found free in the cytoplasm, and had both a plasma membrane and a cell wall. The helical mollicute (2 to 3 μm in length) was blunt at its wide end where it averaged about 260 nm in diameter and contained electron-lucent bodies. Helical turns along its tapered axis resembled those of a spirochete or a spiroplasm (in the class Mollicutes). Krol et al. (1991) also concluded that the helical bacterium did not possess periplasmic flagella, which are characteristic of spirochetes. This apparent absence of flagella and a cell wall in the helical form led Krol et al. (1991) to conclude that the bacterium was some sort of a spiroplasm.

The third morphological form described by Krol et al. (1991) was a filamentous mollicute that appeared as masses of short, branched filaments 60 nm wide with intermittent spherical dilations and terminal blebs on the branches. Further ultrastructural studies done by Krol and co-workers at the same laboratory have led them to conclude that the filamentous mollicute they described was not a microorganism, but that the filamentous forms represented remnants of the host-cell endoplasmic reticulum (Lotz & Overstreet 1991).

In the more recently published morphological study of TNHP, Frelier et al. (1992) confirmed the observations of Krol et al. (1991) that the same 2 morphological forms of intracellular bacteria are present in affected hepatopancreas (HP) cells of shrimp with TNHP. However, Frelier et al. (1992) noted that the helical organism was Gram negative and that it possessed both a cytoplasmic membrane and an outer envelope. Frelier et al. (1992) further noted that these features, along

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with the presence of prominent electron-lucent vacuoles in the helical TNHP agent, are inconsistent with the characteristics of the *Spiroplasma* in the class Mollicutes, which, according to Whitcomb (1980), are Gram positive, do not possess electron-lucent vacuoles, and are bound by only a plasma membrane.

Morphological evidence is presented in this paper that supports the contention that the previously reported 2 principal morphological types, a rickettsia-like form and a helical form, are, in reality, morphological variants of the same bacterium.

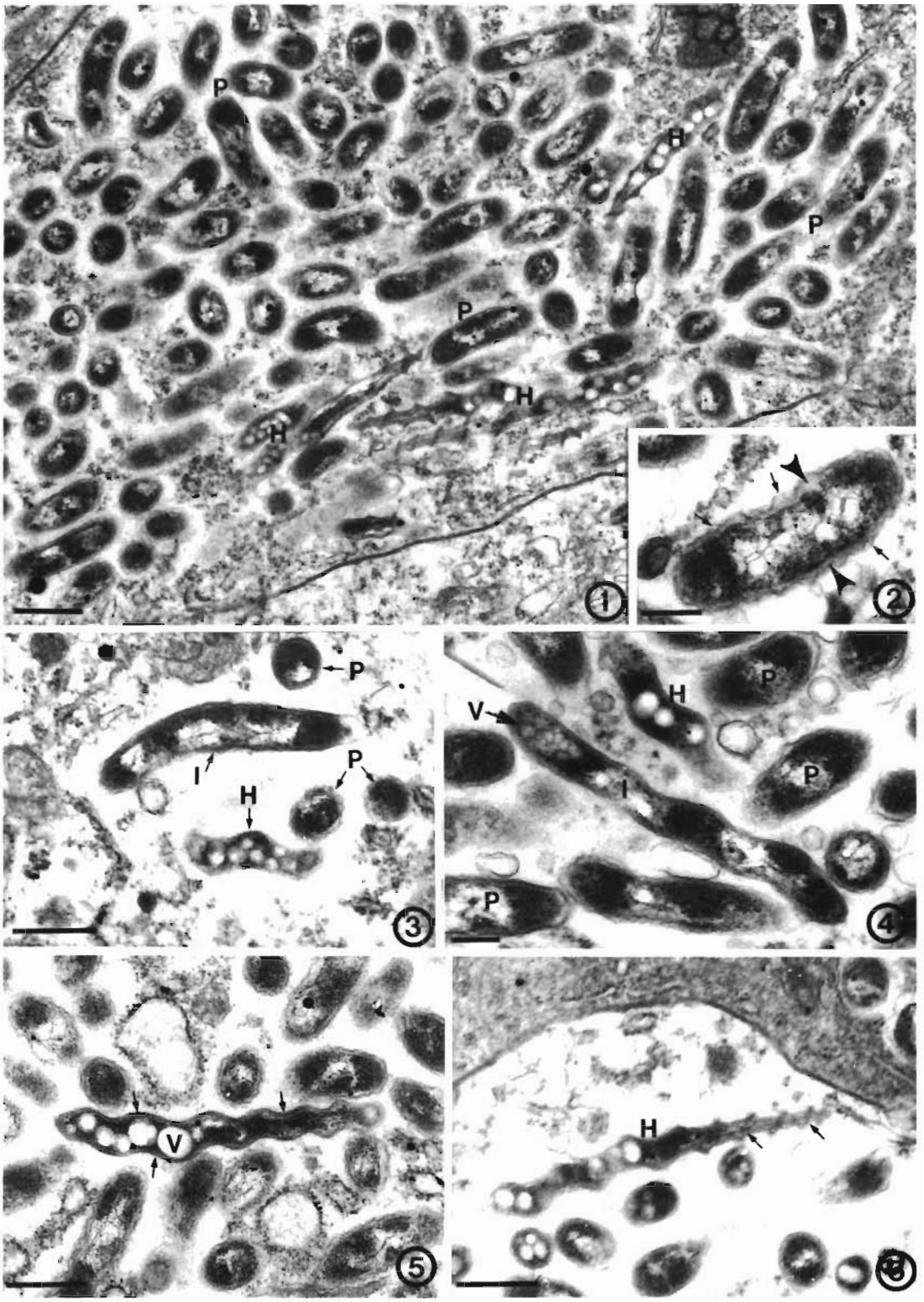
Materials and methods. Two samples of farm-reared juvenile *Penaeus vannamei* from Texas shrimp farms were used in this study. The initial sample set was obtained in July 1990, as Davidson's-preserved (Bell & Lightner 1988) and glutaraldehyde-preserved specimens from a commercial shrimp farm located in south-central Texas near Corpus Christi. The second set of samples was obtained in September 1991, as live specimens from a different shrimp farm that was also located near Corpus Christi. In both samples, affected shrimp were 4 to 6 g juveniles that were clinically diseased or moribund when collected from the shrimp culture ponds.

Specimens for electron microscopic study were fixed live by injection into the HP and adjacent tissues (using a 1 ml syringe) of cold (ca 4 °C) 6 % glutaraldehyde in 0.15 M Millonig's phosphate buffer supplemented with 1 % sodium chloride and 0.5 % sucrose to achieve a final osmolarity of the fixative of ca 750 mmol kg^{-1} as determined with a Wescor 5100C osmometer. Subsequently, the shrimp were immersed whole in the same fixative and the carapace was dissected to enhance fixative penetration. HP's from 5 specimens were subsequently excised and post-fixed in 1 % phosphate-buffered osmium tetroxide, dehydrated, embedded in Spurr's resin (Ladd Research Inc., Burlington, VT), sectioned, stained with lead citrate and uranyl acetate, and viewed using an Hitachi HU-12 operated at 75 kV.

To isolate physically the TNHP helical form of the intracellular bacteria, a heavily infected hepatopancreas was removed from a moribund juvenile, confirmed to be heavily infected using a Giemsa-stained impression smear, and then homogenized in TN (0.02 M tris-HCl, 0.4 M NaCl, pH 7.4) buffer. The HP homogenate was centrifuged at 6000 rpm for 10 min. The pellet was resuspended in TN buffer and centrifuged for 30 min at 16 000 rpm through a layered (15, 30, 40, 50, and 65 %) sucrose discontinuous gradient. Fractions were collected from the centrifuge tube, diluted and pelleted at 6000 rpm for 15 min. A sample of each pellet was transferred to a glass slide, stained with Giemsa, and examined at 1000 × to determine where the helical TNHP organism had concentrated. The bacteria from the richest fractions were deposited onto formvar coated copper grids, negatively stained with 2 % phosphotungstic acid (PTA) at pH 7.0 and viewed by TEM.

Results. A single type of microorganism with multiple morphological or developmental stages was present in the affected tubules of the hepatopancreata of all 5 shrimp HP's examined by TEM. The predominant morphological form (= the 'primary form') of this microorganism was a small, pleomorphic rod-shaped bacterium (Figs. 1 & 2) which averaged 0.25 µm in diameter and ca 0.90 µm in length. This form contained poorly differentiated electron-dense and electron-lucent regions in its protoplasm and was bound by a cell envelope consisting of an inner membrane, a periplasmic space, and an outer often undulant membrane (Figs. 2 to 7). Slightly bent elongated rod-shaped morphological variants of the primary form were relatively common in our preparations (Figs. 1 & 3). Except for its increased length and bent morphology, this 'intermediate form' differs little from the primary form. However, in one end (which will become the basal end) of the intermediate form discrete electron-lucent vacuoles develop and the elongated rod becomes sinuous (Fig. 4). The basal-end vacuoles increase in size, while the torsion of the apical end of the bacterium becomes more pronounced towards the completion of the intermediate stage of development (Fig. 5).

Figs. 1 to 6. TNHP agent from *Penaeus vannemei*. Fig. 1. Low-magnification TEM of a hepatopancreatocyte from a juvenile *Penaeus vannamei* with the TNHP syndrome. Profiles of intracellular rod-shaped or 'primary' (P) forms and 'terminal' or helical (H) forms of bacteria are abundant in the cytoplasm. Scale bar = 0.5 µm. Fig. 2. High-magnification TEM of the rod-shaped 'primary' form of the TNHP agent. Apparent in the bacterium are poorly differentiated electron-dense and electron-lucent regions of protoplasm, a cell envelope composed of an inner membrane and periplasmic space (arrowheads), and an outer undulant membrane (small arrows). Scale bar = 0.2 µm. Fig. 3. Profile of an early 'intermediate form' (I) of the TNHP agent. Only the characteristic flexure of this form distinguishes it from the rod-shaped 'primary form' (P), a few of which are shown in cross section. A portion of the 'terminal' or helical (H) form of the TNHP agent is also present. Scale bar = 0.5 µm. Fig. 4. TEM of a more advanced 'intermediate form' (I) of the TNHP agent. Present at this stage are developing electron-lucent vacuoles (V) in the basal end of the now sinuous bacterium. Also present are several 'primary' (P) forms and one 'terminal' or helical (H) form of the bacterium. Scale bar = 0.2 µm. Fig. 5 Profile of a late 'intermediate' form or an early 'terminal' form of the TNHP agent showing the presence of discrete vacuoles (V) in its basal end, a developing helical morphology, and a well-defined cell envelope (arrows). Scale bar = 0.5 µm. Fig. 6. Profile of a 'terminal' or helical (H) form of the TNHP agent showing the pronounced helical form of the agent and the presence of a tubular structure (arrows) along the apex of the helix. Scale bar = 0.5 µm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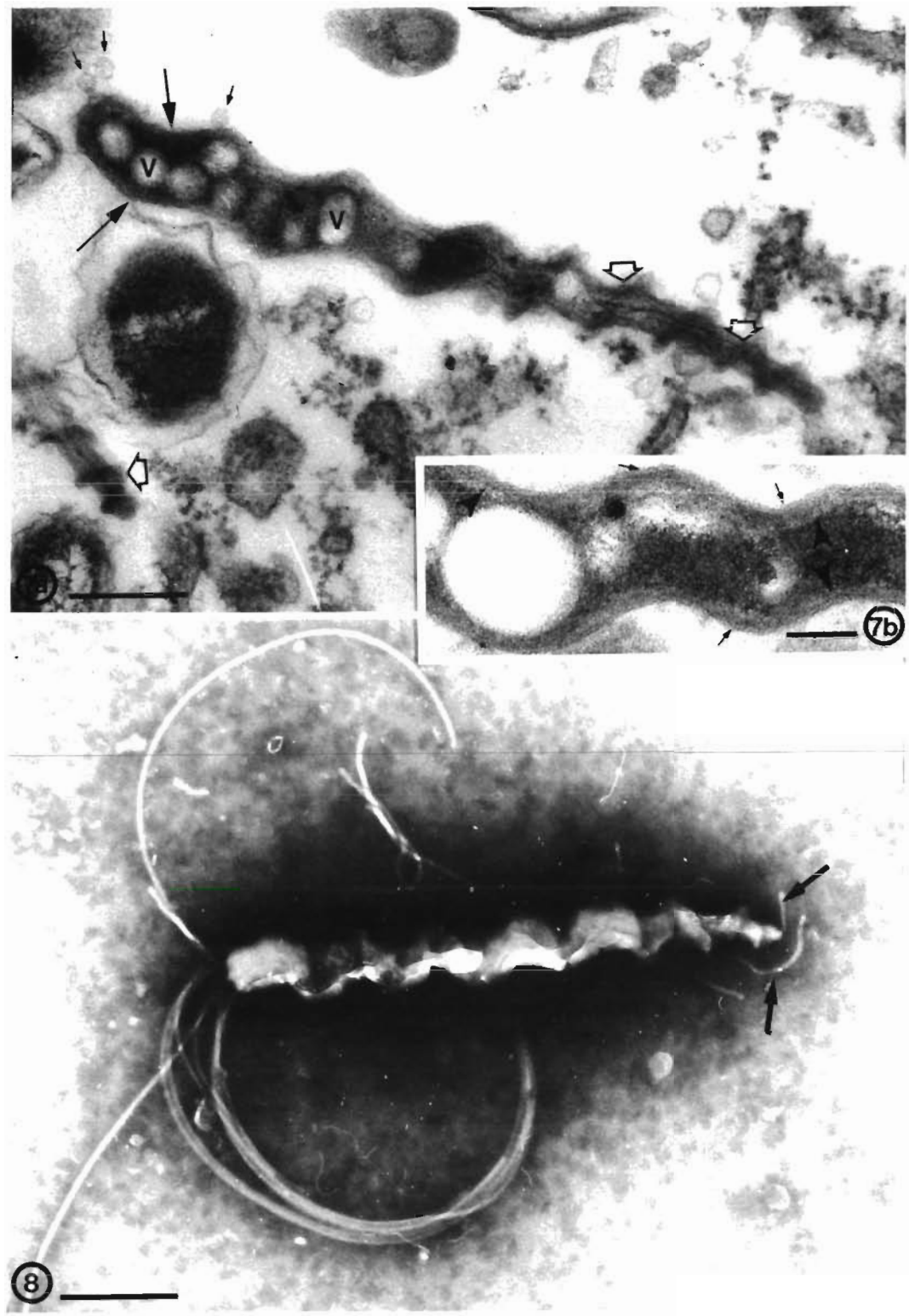

Figs. 7 & 8. TNHP agent from *Penaeus vannamei*. Fig. 7. Higher-magnification TEM's of the 'terminal' or helical form of the TNHP agent. (a) Cell envelope (large arrows) a tubular structure on the apex of the helix (hollow arrows), vacuoles in its basal end (V), and profiles of presumed flagella (small arrows) near its basal end. Scale bar = 0.5 µm. (b) Cell envelope in more detail. It is composed of an inner membrane and periplasmic space (arrowheads), and an outer undulant membrane (small arrows). Scale bar = 0.1 µm. Fig. 8. TEM of an isolated 'terminal' helical form of the TNHP agent. Eight periplasmic flagella arise from the basal end of the bacterium, and other very short flagella (arrows) seem to project from the helix. 2% PTA staining. Scale bar = 0.5 µm

The 'terminal' or helical morphological form of this organism is illustrated in Figs. 6 to 8. This helical form measures ca 0.25 µm in diameter by 2 to 3.5 µm in length, and it possesses numerous vacuoles in its basal end. The conical apical end displays 4 to 10 (in our preparations) prominent helical twists. Present on the apex of the helix is a hollow tubular structure 50 nm in diameter (Figs. 6 & 7), which appears identical to sections of flagella which originate from the basal end of the bacterium (Fig. 7). A cell envelope, consisting of an inner membrane, a periplasmic space, and an outer often undulant membrane, is clearly present surrounding the basal-medial portion of the protoplasm of the helical form of the bacterium (Fig. 7).

The observation of helical forms concentrated by use of a discontinuous sucrose gradient provided more details on the structure of this bacterium (Fig. 8). Isolated helical bacteria measured 2.65 to 2.9 µm in length by 0.25 µm in diameter at their base. The apical portion with its prominent helix narrowed to a fine point after completion of twisting by as many as 10 complete torsions. Eight flagella were found projecting from the basal end of the bacterium. These flagella are presumed to correspond to similarly positioned tubular structures in the sectioned helical bacterium shown in Fig. 7.

Discussion. Morphological characteristics presented here for the agent of TNHP support the theory that a single intracellular bacterium is present in shrimp hepatopancreatocytes with the disease. This is contrary to the conclusions reached in earlier descriptive papers by Krol et al. (1991) and Frelier et al. (1992), in which the morphological forms illustrated in the present paper were considered to represent at least 2 different species of intracellular bacteria. Our findings also support the contention of Frelier et al. (1992) that the helical form is not a spiroplasm belonging to the class Mollicutes, but rather that it is a true Gram-negative bacterium.

Our observations on the morphological stages of the TNHP agent may aid the eventual taxonomic placement of this unique bacterium. While further studies on the organism will be needed to classify it correctly, we note that this bacterium may be related to members of the genus *Seliberia*, with which it shares a few significant morphological characteristics (Schmidt & Starr 1989). Among the shared characteristics of the TNHP agent and *Seliberia* are: both have rod- (or ovoid-) shaped and helical morphological stages; both have periplasmic flagella; and both have discrete intracellular electron-lucent vacuoles (which contain hydroxybutyric acid in *Seliberia* spp.) (Kutz 1987, Schmidt & Starr 1989). While the similarities of the TNHP agent and *Seliberia* spp. are intriguing, all of the known species of *Seliberia* occur in soil and freshwater environments as autochthonous microflora and none are known to be pathogenic (Schmidt & Starr 1989).

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