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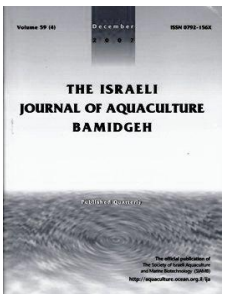

Histopathology of *Spiroplasma penaei* Systemic Infection in Experimentally Infected Pacific White Shrimp, *Penaeus vannamei*

Allan Heres*, Rita Redman, Donald V. Lightner

Department of Veterinary Science and Microbiology, University of Arizona, 1117 E. Lowell, Tucson, Arizona 85721, USA

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Abstract

Penaeus vannamei shrimp were challenged with a suspension of a pathogenic isolate of *Spiroplasma penaei* prepared from a 72-h culture. The route of challenge was by intramuscular injection of the bacterial suspension into the third abdominal segment. Lesion development was evaluated in moribund shrimp collected and fixed in Davidson's fixative 96 h post challenge. The predominant host responses to infection by *S. penaei* observed by histological examination were the general systemic development of hemocytic nodules (often melanized) and poorly organized hemocytic infiltration. Such lesions were most prevalent in the lymphoid organ, gill filaments, heart, connective tissue, antennal gland, and skeletal muscle. The presence of *S. penaei* in the lesions was verified by *in situ* hybridization using a digoxigenin (DIG)-labeled probe specific to the spiralin gene of *Spiroplasma* spp. Transmission electron micrographs (TEM) showed *S. penaei* cells free in the cytoplasm of lymphoid organ cells. The cultures of *S. penaei* used for this study and infected abdominal tissue were verified by PCR using spiroplasma-specific primers that amplify a fragment from a variable region of the 16S rDNA gene sequence.

* Corresponding author. E-mail: aaheres@aol.com

Introduction

The first pathogenic spiroplasma to be found in marine shrimp was described by Nunan et al. (2004). The disease agent was isolated from the hemolymph of the Pacific white shrimp, *Penaeus vannamei*, raised in a shrimp farm with very low salinity brackish water that was suffering from high mortalities. Histological analysis detected systemic inflammatory reactions in affected organs/tissues. The organism was found to be pleomorphic, but often helical in shape, and variable in length. *Spiroplasma penaei* is serologically different from other spiroplasma species. Electron microscopy reveals bacteria with a single cytoplasmic membrane and no cell wall (Nunan et al., 2005).

The epidemic of tremor disease in Chinese mitten crabs, *Eriocheir sinensis*, which is an important species in freshwater aquaculture in China, has resulted in great economic losses (Wang et al., 2004). Infected crabs display signs of weakness, anorexia, intense tremors, and death. The agent described as *Spiroplasma* spp. was found in hemocytes, muscles, nerves and connective tissues of cardiac and pereiopod muscles. A systemic infection of spiroplasmas in the red swamp crayfish, *Procambarus clarkii*, in the summer of 2004 in freshwater aquaculture in China was described by Wang et al. (2005). The sick crayfish were in the same ponds as the Chinese mitten crab that were affected by tremor disease. Healthy crayfish were experimentally infected by injection with hemolymph from diseased crayfish or a cultured isolate in M1D media, and by cohabitation with diseased crayfish. The spiroplasmas were detected by transmission electron micrographs (TEM) in the hemolymph, connective tissues of the gonads, pereiopods, gut, hepatopancreas, nerves, heart, and gills.

The histopathology of experimentally induced infections of *Spiroplasma taiwanense* in *Anopheles stephensi* mosquitoes was described by Phillips and Humphery-Smith (1995). Light microscopy showed extensive degradation of the thoracic flight muscle in sections stained with hematoxylin and eosin (H&E) and polysaccharide depletion in sections stained with periodic acid-Schiff. TEM showed spiroplasmas in the hemolymph, and both extra and intracellular spiroplasmas in the thoracic flight muscle, glial cells, neural lamella, hemocytes, connective tissue surrounding the diverticulum and midgut, trophocytes, and tracheocytes. Nerve cord axons surrounded by infected glial cells were distended by swollen mitochondria. The pathologies within the thoracic flight muscle were attributed to intracellular replication of spiroplasma bacterial cells.

Materials and Methods

Bacteria isolate. A pathogenic *S. penaei* isolate, obtained in pure form by filtration and limiting dilution, was isolated by Nunan et al. (2004) from the hemolymph of moribund Pacific white shrimp, *P. vannamei*, that originated from a shrimp farm near Cartagena, Colombia. The culture had been stored at -70°C in M1D media supplemented with 2% NaCl until used in this study. A single pathogenic isolate was used in its logarithmic phase, indicated by a color change caused by acidification of the culture media; it is referred as the reference isolate in this study.

Bacterial suspension. The bacterial suspension of the *S. penaei* reference isolate was prepared by removing one ml of culture media during the logarithmic phase (~72 h at 28°C) and centrifuging it at 16,300 × *g* for 3 min. The supernatant was discarded and the bacterial pellet was resuspended to a final 1-ml suspension with sterile 2% saline solution. The final working bacterial suspension was adjusted to a final dilution of 1:100 with a sterile 2% saline solution. A sample of ten shrimp received a single injection of 100 µl of the bacterial suspension into their third abdominal segment.

Histology. A sample of three moribund shrimp were fixed with Davidson's fixative 96 h post challenge, transferred to 70% alcohol, later embedded in paraffin, and sectioned for routine hematoxylin and eosin (H&E) histological analysis using standard methods according to Lightner (1996), and the *in situ* hybridization (ISH) technique according to Poulos et al. (1994). Consecutive histological sections were stained with H&E for routine histology and assayed by ISH. The ISH assay for *S. penaei* was developed by Nunan et al. (2004) and uses a digoxigenin (DIG)-labeled gene probe specific to the spiralin gene,

which expresses a protein present in the membrane of *Spiroplasma* spp., to verify the presence of *S. penaei* in the bacterial lesions observed in histological sections.

Transmission electron microscopy (TEM). Lymphoid organs were removed from a sample of three moribund shrimp and placed in 1 ml of 6% buffered glutaraldehyde, prepared with 0.15 M Millonig's phosphate buffer (pH 7.0) and supplemented with 1% NaCl and 0.5% sucrose (Lightner, 1996) for TEM. Following overnight refrigeration (4°C), the buffered glutaraldehyde was removed and replaced with cold Millonig's phosphate buffer (0.15 M) and maintained at 4°C until post-fixation. The tissues were post-fixed with 1% phosphate-buffered osmium tetroxide, dehydrated in ethyl alcohol, and embedded in Spurr's resin (Ladd Research Inc.). The embedded tissues were sectioned to a thickness of 75-90 nm and stained with lead citrate and uranyl acetate. The grids were examined using a Phillips CM12 transmission electron microscope operated at 80 kV.

Negative staining preparation for TEM. Negative staining for TEM is described by Nunan et al. (2004). A drop of resuspended bacterial pellet was placed on a clean piece of parafilm. A clean Formvar/carbon coated copper grid was placed on the surface of the drop for 3 min. The grid was transferred and placed on the surface of a drop of 2% aqueous phosphotungstic acid (PTA), pH 7.0, for 3 min. The grid was air-dried for several hours and examined using a Phillips CM12 transmission electron microscope operated at 80 kV.

Aquaria preparation. Aquaria of 90-l capacity filled with artificial marine water (Crystal Sea Marine-Mix, Marine Enterprises International, Baltimore, Maryland) at 25 ppt salinity and 28°C were prepared.

Shrimp. Specific pathogen free (SPF) *P. vannamei* shrimp with an average weight of 2 g (Lightner and Redman, 2009), which originated from the Oceanic Institute in Hawaii, were used in this study. Shrimp were fed once daily *ad libitum* with a commercial pelleted feed (Rangen, 35% protein, Buhl Idaho).

Results

The predominant host inflammatory responses observed in H&E-stained histological sections from shrimp infected by injection with *S. penaei* were hemocytic nodules, hemocytic infiltration, and melanization of many of the hemocytic lesions. The lesions were generally systemic and often observed in lymphoid organs, gill filaments, hearts, antennal glands, ganglia, skeletal muscles, posterior caeca, and hindguts (Figs. 1, 2). Similar lesions were detected in connective tissues and nerve cords. The presence of *S. penaei* in the lesions observed in histological sections was verified by ISH using a DNA probe specific to the spiralin gene of *Spiroplasma* spp.

TEM showed *S. penaei* without cell walls, free in the cytoplasm of lymphoid organ cells (Fig. 3). The *S. penaei* cells in the lymphoid organ presented several shapes including a helical form. TEM of negatively-stained bacterial suspension from an inoculated media showed filamentous morphology with a vesicular bleb and a single cytoplasmic membrane without a cell wall (Fig. 4).

Discussion

The lesions observed by light microscopy were generally the systemic development of hemocytic nodules (often melanized) and poorly organized hemocytic infiltration. Such lesions were most prevalent in the lymphoid organ, gill filaments, heart, connective tissue, antennal gland, and skeletal muscle. Transmission electron micrographs showed free *S. penaei* cells in the cytoplasm of lymphoid organ cells.

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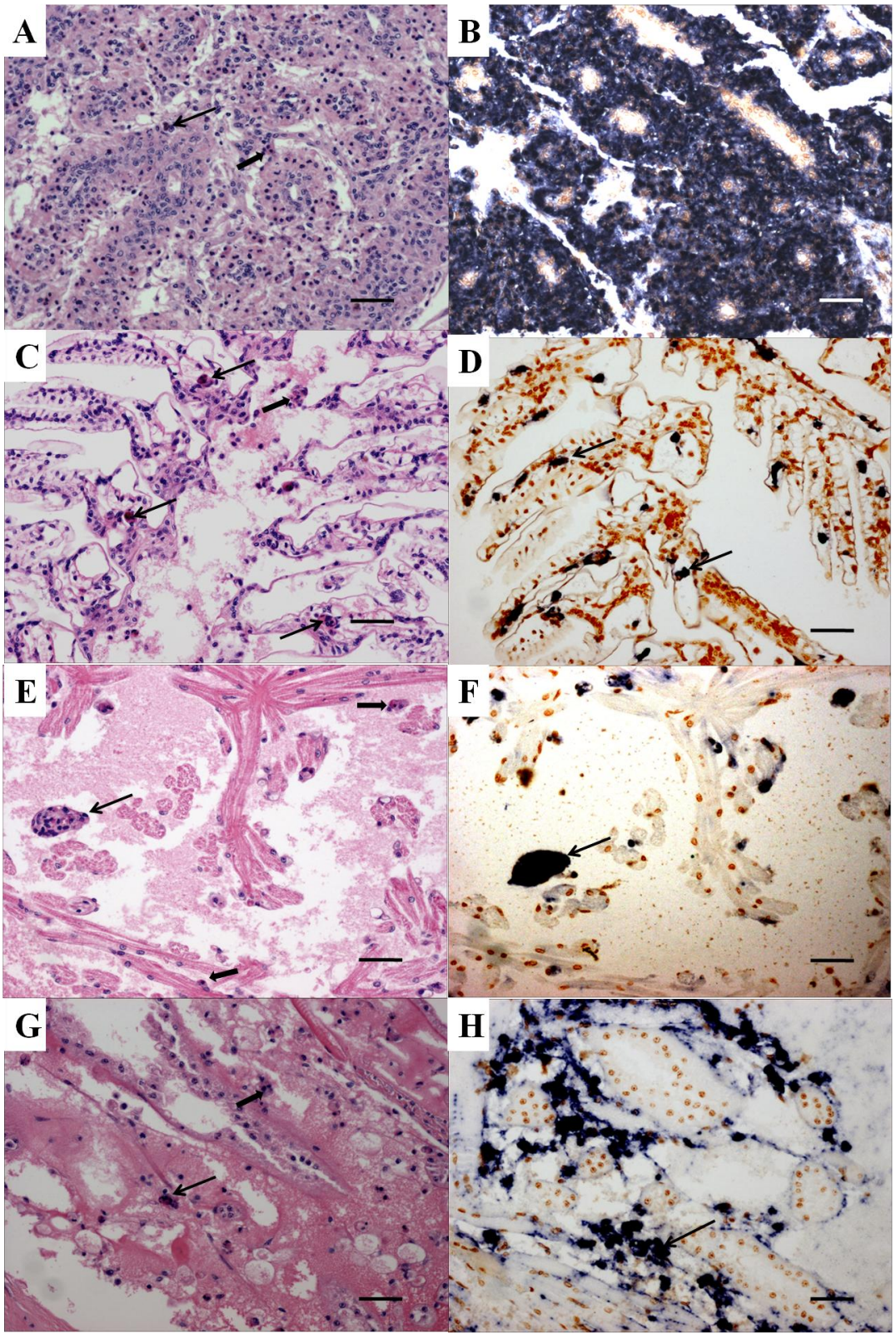

Fig. 1. Photomicrographs of H&E-stained (left column) and ISH-assayed (right column) tissue sections of *Penaeus vannamei* injected with a bacterial suspension of pathogenic *Spiroplasma penaei*. Bacterial lesions and positive ISH reactions are indicated by blue-black precipitates in (A, B) lymphoid organ, (C, D) gill filaments, (E, F) heart, and (G, H) antennal glands. Hemocytic nodules are indicated by narrow arrows and hemocytes with pyknotic nuclei by broad arrows. Scale bars = 50 µm.

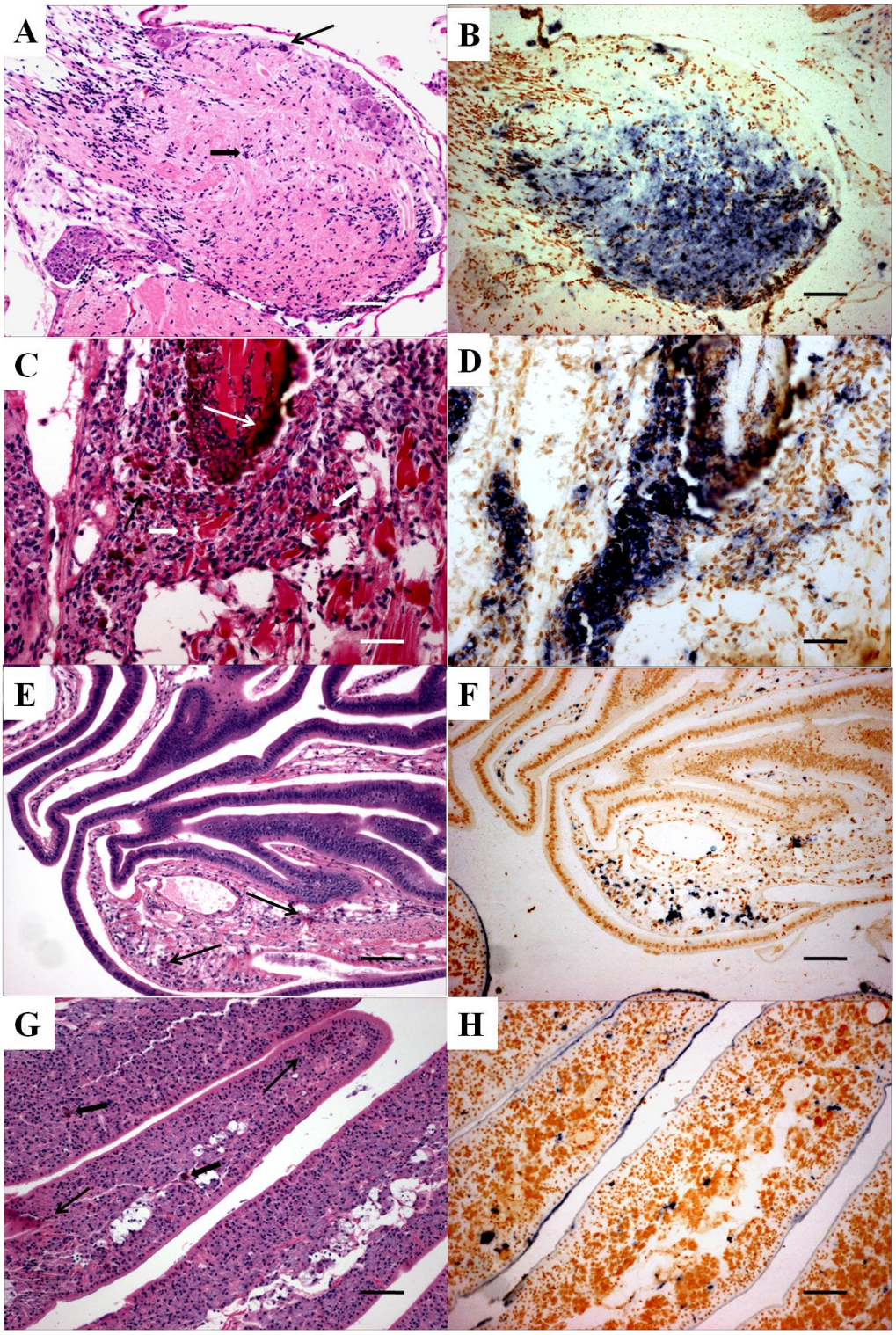

Fig. 2. Photomicrographs of H&E-stained (left column) and ISH-assayed (right column) tissue sections of *Penaeus vannamei* injected with a bacterial suspension of pathogenic *Spiroplasma penaei*. (A, B) ganglion, (C, D) skeletal muscle, (E, F) posterior midgut caecum, and (G, H) hindgut. Bacterial lesions and positive ISH reactions are indicated by blue-black precipitates. Hemocytic nodules and hemocytes with pyknotic nuclei are indicated by narrow arrows and broad arrows, respectively, in plates A, E, and G. Hemocytic infiltration and melanization are indicated by narrow arrows, and a fragment of necrotic, hemocyte inflamed skeletal muscle is indicated by broad arrow in plate C. Scale bars = 50 μm.

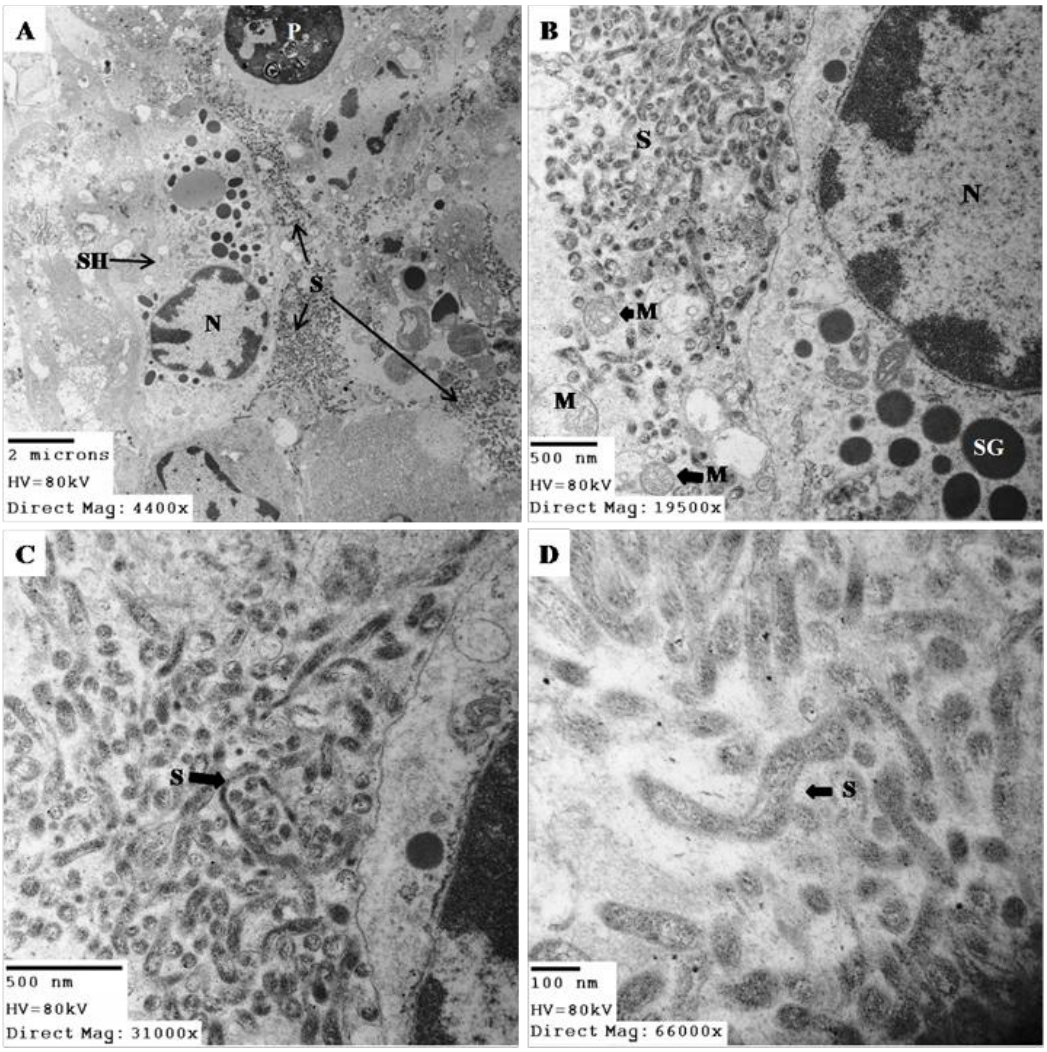

Fig. 3. Transmission electron micrographs of *Spiroplasma penaei* in the cytoplasm (arrows) of lymphoid organ cells of *Penaeus vannamei* injected with a bacterial suspension of pathogenic *Spiroplasma penaei*. M = mitochondrion, N = nucleus, P = phagolysosome, S = spiroplasma, SG = secretory granule, SH = semigranular hemocyte. Scale bar = 2 µm (A), 500 nm (B), 500 nm (C), 100 nm (D).

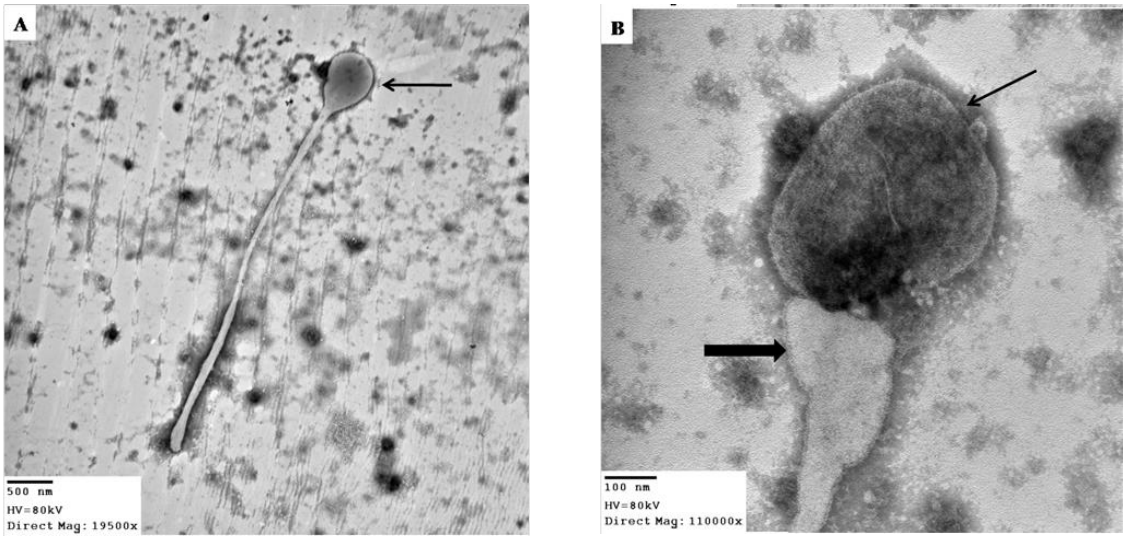

Fig. 4. Transmission electron micrographs of *Spiroplasma penaei* from inoculated M1D media after 72 h at 28°C. Negatively stained bacterial suspension shows filamentous morphology and a vesicular bleb (narrow arrows). A single cytoplasmic membrane (broad arrow) is depicted and a cell wall is absent. Scale bar = 500 nm (A), 100 nm (B).

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