

Experimental infection of *Penaeus vannamei* by a rickettsia-like bacterium (RLB) originating from *P. monodon*

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ABSTRACT: A rickettsia-like bacterium (RLB), which caused severe mortalities of commercially farmed *Penaeus monodon* in the southwest region of Madagascar, was investigated to determine whether the organism would produce the same disease in *P. vannamei*. Two series of bioassays were performed to determine whether this RLB could be transmitted to *P. vannamei* through injection and per os exposure. The first series of challenge bioassays used frozen, RLB-infected *P. monodon* tissue from Madagascar as the inoculum and feed for the injection, and per os bioassays with specific pathogen free (SPF) *P. vannamei*. In the second series of bioassays, frozen RLB-infected *P. vannamei* tissue derived from the first series of injection bioassays was used as the inoculum to challenge by injection and per os SPF *P. vannamei*. Disease status was determined through standard histological techniques and by *in situ* hybridization assays with a digoxigenin-labeled probe specific for this RLB. The results indicated that *P. vannamei* did develop the RLB infection when injected with either RLB infected *P. monodon* or *P. vannamei* tissue homogenates. This contrasts with results from the per os exposure to the RLB in which the disease could not be reproduced.

KEY WORDS: Rickettsia · *Penaeus monodon* · *Penaeus vannamei* · Shrimp

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INTRODUCTION

In the southwest region of Madagascar in the fall of 1999, severe mortalities of commercially farmed *Penaeus monodon* occurred in grow-out ponds. Clinical signs of diagnostic importance included necrosis on the carapace of moribund animals, and some of the shrimp displayed calcium precipitate visible on the cephalothorax. Although no grossly visible atrophy was observed, moribund shrimp in the pre-molt stage were characterized by a whitish hepatopancreas. Histological examination of moribund shrimp samples preserved in Davidson's fixative (Bell & Lightner 1988) revealed a systemic infection caused by a rickettsia-like bacterium (RLB). Cytoplasmic masses were composed of tiny bacterial rods of ~0.5 to ~1 µm that stained gram-negative with the Gram-Twort stain (Ollett 1947). The principal target tissues affected by

the RLB in *P. monodon* included, in order of severity, the lymphoid organ, connective tissue (systemically), circulating hemocytes, fixed phagocytes, and cuticular epithelial cells (Nunan et al. 2003).

A wide range of aquatic crustaceans can be infected with rickettsia-like microorganisms. Amphipods, crabs, freshwater crayfish and prawns have been documented to be infected with RLB (Federici et al. 1974, Harshbarger et al. 1977, Bonami & Pappalardo 1980, Larrison 1982, Johnson 1984, Brock et al. 1986, Anderson et al. 1987, Krol et al. 1991, Frelier et al. 1992, Fryer & Lannan 1994, Bower et al. 1996, Jimenez et al. 1997, Edgerton & Prior 1999, Wang & Gu 2002, Nunan et al. 2003). The purpose of this study was to determine whether the RLB that caused severe mortalities in *Penaeus monodon* could infect and cause mortalities in specific pathogen free (SPF) *P. vannamei*. With the international movement of shrimp stocks (Lightner et

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al. 1997, Harper 2002), and the co-culture of *P. monodon* and *P. vannamei* in grow-out ponds in areas of Southeast Asia (Lightner et al. 1987, Rosenberry 2000), the infectivity of this RLB in *P. vannamei* becomes an important cultural and economic consideration.

MATERIALS AND METHODS

Experimental shrimp. Specific pathogen free (SPF) *Penaeus vannamei* were obtained from the Oceanic Institute in Hawaii (Wyban et al. 1992, Pruder et al. 1995). These shrimp were reared from postlarvae to approximately 3 g juveniles at the University of Arizona Aquaculture Pathology Center using the methods reported by White et al. (2002). In each of the bioassays performed, 4 × 90 l capacity glass aquaria were stocked with 5 shrimp per tank. Shrimp were acclimated for 24 h prior to treatments. Both injection bioassays were conducted for a period of 21 d. The per os bioassays were maintained for a minimum of 21 to 35 d. Injection bioassay shrimp were fed a daily ration of the commercial Rangen 35% pellet diet at a rate of 4% of their overall biomass. Per os bioassay shrimp were fed either minced *Penaeus monodon* tissue at a rate of 5% of the overall biomass for 2 consecutive days or minced *P. vannamei* tissue at a rate of 5% of the overall biomass for 5 consecutive days.

The aquaria were disinfected (200 ppm chlorine from calcium hypochlorite), freshwater rinsed and filled with artificial seawater (salinity 23 ppt) (Forty Fathoms Bio-Crystals Marinemix, Marine Enterprises International) and maintained at room temperature (~25°C). Each aquarium was outfitted with a 1 l biological filter containing crushed oyster shell and granular activated carbon, with water recirculation provided by an airlift to each filter. Aeration was delivered to each tank by 2 teflon air lines: 1 for the airlift and 1 for water aeration. Control aquaria were isolated from the treatment aquaria and the top of each was covered with a plastic sheet to prevent aerosol cross-contamination. Individual nets were assigned to each tank to maintain sanitary conditions.

Inoculum preparation and injection. Both inocula for the injection bioassays were prepared by homogenizing 1 g of infected tissue, originating from either naturally infected *Penaeus monodon* or experimentally infected *P. vannamei*, in 1 × TN (Tris; NaCl). The homogenates were centrifuged at 3000 × *g* for 10 min at room temperature, to pellet cellular debris. The supernatant fluid was diluted in sterile 2% NaCl to a final dilution of 1:50. Treatment shrimp were intramuscularly injected (0.05 ml) in the third abdominal segment using a sterile 1 ml syringe fitted with a 25 gauge 16 mm needle. The negative control shrimp were injected with sterile 2% NaCl. Prior to initiation of the

bioassays, 3 randomly selected shrimp from the SPF population were preserved in Davidson's fixative and their SPF status was verified by routine histology following standard methods.

Bioassay 1A. Injection of rickettsia infected *Penaeus monodon* homogenate into *P. vannamei*: The inoculum was prepared from frozen (-70°C) RLB-infected *P. monodon*, which originated from a shrimp farm in the southwest region of Madagascar collected in the fall of 2000 (Nunan et al. 2003). A total of 15 SPF *P. vannamei* were injected with the inoculum and then equally distributed between the 3 aquaria. In addition, 5 SPF shrimp were injected with saline (2% NaCl) and served as the negative control treatment. The shrimp were observed daily for signs of disease. During the course of the bioassay, moribund shrimp and representative survivors were preserved in Davidson's fixative (Bell & Lightner 1988). Records of daily mortalities were maintained. Dead shrimp, when observed, were removed from the aquaria and disposed of.

Bioassay 1B. Injection of rickettsia infected *Penaeus vannamei* homogenate into *P. vannamei*: From the previous bioassay, 1 g of frozen RLB-infected *P. vannamei* tissue was used for the inoculum in this series of bioassays. SPF *P. vannamei* (15) were injected with the inoculum, equally distributed among aquaria and observed daily for signs of disease. The negative control for this series of bioassays was 5 SPF shrimp injected with saline. Moribund shrimp were collected and fixed in Davidson's fixative during the course of the experiments to confirm disease status.

Bioassay 2A. Per os exposure of *Penaeus monodon* to rickettsia infected *P. vannamei* tissue: RLB-infected minced *P. monodon* tissue (Nunan et al. 2003) was fed at a rate of 5% total body mass to 15 SPF *P. vannamei*. Approximately 4.5 g of the tissue, originating from frozen shrimp carcasses from the rickettsial disease outbreak in Madagascar, was fed to the indicator SPF shrimp at 2.5% body weight in 2 feedings daily for 2 d. The 3 replicate tanks were maintained on pelleted rations following the per os exposure. In addition, 5 SPF shrimp were fed pelleted rations and these acted as the negative control for this series of bioassays.

Bioassay 2B. Per os exposure of *Penaeus vannamei* to rickettsia infected *P. vannamei* tissue: Approximately 12 g of RLB-infected *P. vannamei* carcasses collected on Days 11 through 14 during bioassay 1B was minced and 750 mg (2.5% body mass) was placed into separate bags for twice daily feedings of 15 SPF *P. vannamei*. Per os exposure in this series of bioassays was 5 d. Following the exposure to infected tissue, the shrimp were fed a pelleted ration. One 90 l tank contained 5 SPF shrimp which were fed only a pelleted ration for the duration of the bioassay and were maintained as the negative control.

Histology and gene probe. Moribund and surviving shrimp from each of the bioassays were preserved in Davidson's fixative for histological examination (Bell & Lightner 1988) and *in situ* hybridization assays with a digoxigenin-labeled DNA gene probe specific to this RLB (Nunan et al. 2003).

RESULTS

Time course of mortality in the injection bioassays

In Bioassay 1A, mortalities began at 5 d post-injection (PI) and continued through 19 d PI. A total of 57% of the mortalities occurred from 13 d to 15 d PI. Cumulative mortalities ranged between 80 to 100% among each treatment group. No mortalities occurred in the SPF negative control populations injected with saline (Fig. 1A).

Bioassay 1B revealed similar mortality data when compared with Bioassay 1A. Mortalities began 8 d PI and continued through 12 d PI, with 60% of the die-off occurring at 9 and 10 d PI. Cumulative mortalities of the 3 replicate treatment groups was 100%. No mortalities occurred in the SPF negative control group that was injected with saline (Fig. 1B).

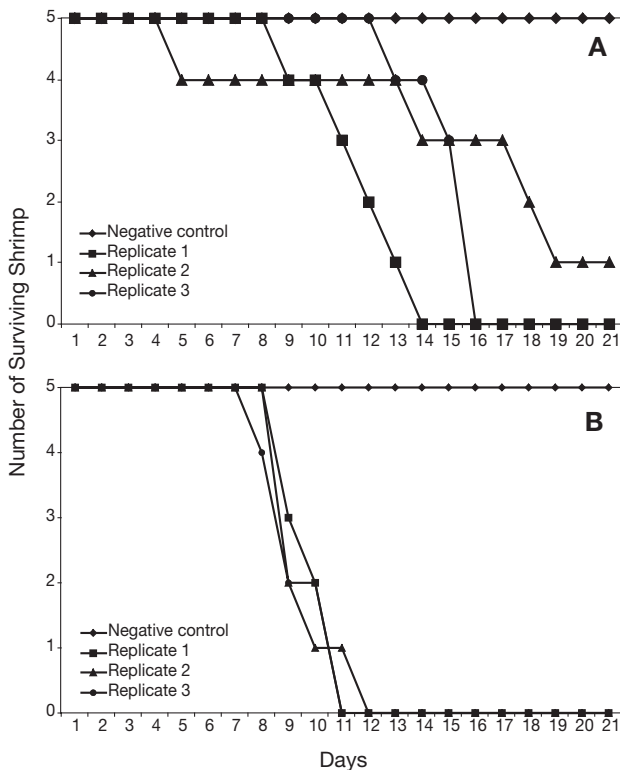


Fig. 1. Mortality results from the injection challenges in which (A) *Penaeus monodon* rickettsia-like bacterium (RLB)-infected tissue homogenate was injected into specific pathogen free (SPF) *P. vannamei*, and (B) *P. vannamei* RLB-infected tissue homogenate was injected into SPF *P. vannamei*

The per os bioassays did not demonstrate infectivity of this organism by this route of exposure. In Bioassay 2A, 1 mortality occurred 6 d PI. There were 14 surviving shrimp when the experiment was terminated 21 d PI. As in the injection bioassays, no mortalities occurred in the SPF control group (Fig. 2A).

Bioassay 2B yielded similar results to Bioassay 2A. In this per os experiment, the indicator *P. vannamei* shrimp were fed 5% of their body weight for 5 d and monitored for 35 d. The negative results from Bioassay 2A necessitated increasing the RLB-infected feed and the length of the experiment. Even changing these parameters from the first per os bioassay did not produce infection. One mortality in the treatment groups occurred 27 d PI. 14 shrimp survived until 35 d PI when the experiment was terminated. All of the SPF shrimp survived (Fig 2B).

Histological and gene probe confirmation of RLB infection

Histological examination revealed RLB infections in shrimp sampled from the injection bioassay experiments. The principal target tissues affected by the RLB included, in order of severity, the lymphoid organ (LO),

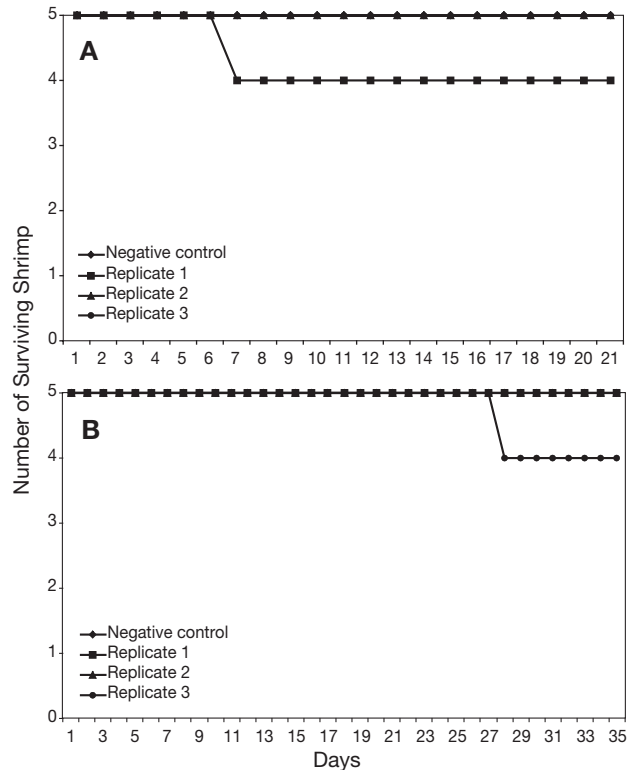


Fig. 2. Mortality results from per os challenges in which (A) *Penaeus monodon* rickettsia-like bacterium (RLB)-infected tissue was fed at 5% body weight for 2 d to specific pathogen free (SPF) *P. vannamei*, and (B) *P. vannamei* RLB-infected tissue was fed at 5% body weight for 5 d to SPF *P. vannamei*

connective tissue (systemically), and heart and gill appendages. This organism did not infect the mucosal epithelial cells of the midgut, anterior midgut caecum or hepatopancreas. The degree of infection in the target tissues was variable. Cytoplasm of some infected cells was filled with the organism, while the cytoplasm of adjacent cells contained far fewer RLB.

In situ hybridization assays, a useful molecular tool to further delineate the affected tissues, showed strong, positive signals only in tissues of mesodermal origin. The order of severity was similar to that observed by histological examination with the LO, typically the most severely affected in the experimental injection infections (Fig. 3). The gill and heart also

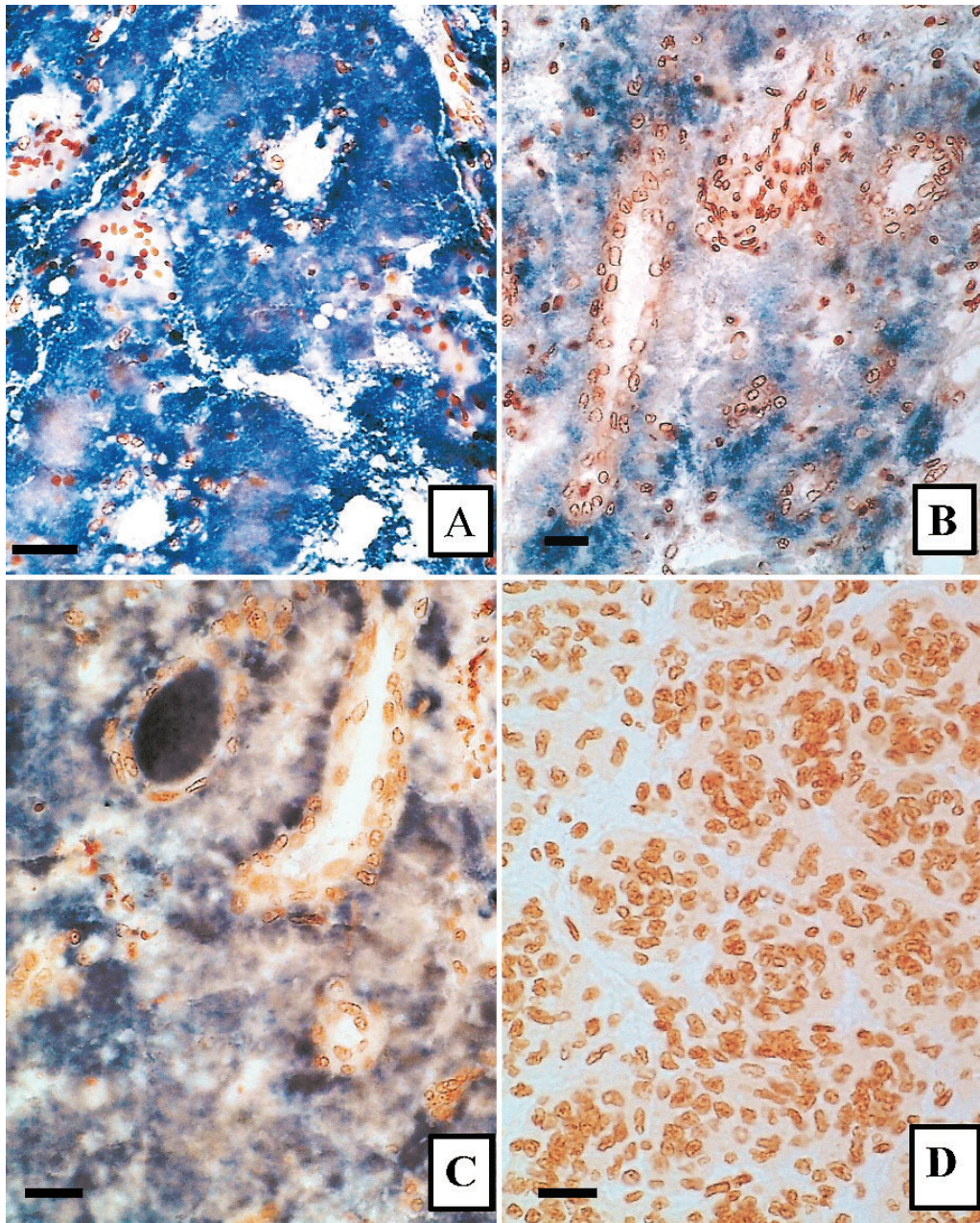


Fig. 3. *In situ* hybridization assay results in the lymphoid organ (LO) stomal matrix cells using a digoxigenin-labeled DNA gene probe specific to the rickettsia-like bacterium (RLB). A positive hybridization signal is demonstrated by the dark purple precipitate. (A) Naturally occurring RLB infection in *Penaeus monodon*, (B) experimentally induced RLB infection in *P. vannamei* using a RLB infected *P. monodon* homogenate, (C) experimentally induced RLB infection in *P. vannamei* using a RLB infected *P. vannamei* homogenate, and (D) no reaction observed in the per os challenge using RLB infected *P. monodon* tissue fed to *P. vannamei*. Scale bars = 20 μ m

presented strong probe signals. A probe signal was also present in the striated muscles, heart, the hemopoietic nodules, nerve ganglia, and nerve cord. However, these tissue types were not infected because the positive probe reaction was a result of the presence of infected connective tissue cells or hemocytes in these tissues. SPF shrimp, sampled from the negative control aquaria at the beginning and at the termination of the bioassay experiments, did not react to the probe. Additionally, the shrimp tissues from the per os experiments did not react to the RLB probe (Fig. 3).

DISCUSSION

The results from previous studies in aquatic crustaceans infected with rickettsia or rickettsia-like organisms have demonstrated that these bacteria can cause, or are suspected of causing, significant losses in commercially raised shrimp, crayfish and wild crabs. With the movement of aquatic species from one geographical area to another, the possibility of an infectious agent in *Penaeus monodon* being spread to areas that culture *P. vannamei* becomes an important economic consideration. The disease caused by white spot syndrome virus (WSSV) first severely impacted the Asian shrimp growing regions and affected farms raising *P. japonicus*, *P. chinensis* and *P. monodon*. A few years later, WSSV was found in *P. vannamei*, *P. setiferus* and *P. stylirostris* in the Western Hemisphere and caused similar, severe mortalities as were seen in Asian penaeids (Lightner et al. 1997). The purpose of the present study was to determine whether the RLB can infect *P. vannamei* and cause similar disease and mortalities.

Exposure of SPF *Penaeus vannamei* to the RLB was accomplished by 2 methods. The first treatment was by injection of an RLB-infected homogenate into the SPF indicator shrimp. Cumulative mortalities of 97% occurred in the shrimp challenged by injection. This contrasts with the results from the per os exposure of SPF *P. vannamei* to the RLB, in which there was a 93% survival rate.

The results of these experiments indicate that per os exposure of infected shrimp to SPF *Penaeus vannamei* did not transmit disease. Per os exposure, at the feeding rates investigated in the bioassays, may not have been adequate to result in the transmission of the disease. Age of the indicator SPF shrimp (juveniles) versus the adult *P. monodon* from which the organism originated may also play a role in disease outbreak by per os exposure. Environmental factors, such as seasonally elevated salinity and water temperatures during the grow out season, may affect the per os susceptibility of the affected shrimp. In the experiments

reported here, the salinity and water temperatures were constant throughout the series of bioassays and the experimental shrimp were not subjected to the variations in temperature (>30°C) and salinity (>30 ppt) experienced by the *P. monodon* in their natural environment. The shrimp in the experimental exposures were not in a stressful situation compared with the natural environment in which the *P. monodon* in Madagascar were cultured, and this may have affected per os disease expression.

Another possibility that may explain the per os results is that a parasite or aquatic species, such as crabs, may have served as a vector of the disease. This is another possible route by which transmission of the RLB could be accomplished in the natural environment. In the experimental challenge studies, vectors were not a factor.

In both of the injection bioassays, the SPF shrimp died due to RLB exposure. Disease status was confirmed by histology and *in situ* hybridization assays with a specific gene probe to the RLB. No mortalities were attributed to RLB in the per os challenges. In summary, these series of experiments indicated that *Penaeus vannamei* were susceptible to infection from the RLB that was present in the *P. monodon*. Infectivity data from one shrimp species to another is important information to consider when transporting exotic species from one area of the world to a different geographical location. Importation of live shrimp from zones where major economic diseases are present is a method by which new pathogens can be introduced into new environments and species. Through the use of domestication of shrimp lines and sanitary control, spread of exotic pathogens from one species to another can be minimized.

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