Aquaculture 318 (2011) 273–276

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Evaluation of different microalgae species and Artemia (*Artemia franciscana*) as possible vectors of necrotizing hepatopancreatitis bacteria

Luz Angélica Avila-Villa [a], Marcel Martínez-Porchas [a], Teresa Gollas-Galván [a,*], José A. López-Elías [b], Lauro Mercado [b], Álvaro Murguia-López [b], Fernando Mendoza-Cano [c], Jorge Hernández-López [c]

[a] Centro de Investigación en Alimentación y Desarrollo, A.C. Km. 0.6 Road to La Victoria, Hermosillo, Sonora, México
[b] Departamento de Investigaciones Científicas y Tecnológicas de la Universidad de Sonora. Luis Donaldo Colosio, S/N. Colonia Centro, Hermosillo, Sonora, México
[c] Centro de Investigaciones Biológicas del Noroeste, Avenida Centenario # 53, Hermosillo, Sonora, 83000, México

ARTICLE INFO

Article history:
Received 20 April 2011
Received in revised form 20 May 2011
Accepted 26 May 2011
Available online 17 June 2011

Keywords:
Artemia
NHPB
Pathogen
Shrimp
Microalgae

ABSTRACT

Microalgae species commonly used in shrimp aquaculture and observed in shrimp habitat (*Chaetoceros muelleri*, *Dunaliella* sp. and *Navicula* sp.), and Artemia (*Artemia franciscana*) were evaluated as possible vectors of necrotizing hepatopancreatitis bacteria (NHPB). Microalgae species and Artemia were exposed to NHPB. Eight days after inoculation, NHPB were detected (by PCR) only in *Navicula* sp. Thereafter, the three inoculated microalgae were used to feed white shrimp (*Litopenaeus vannamei*): shrimp fed on *C. muelleri* and *Dunaliella* sp. resulted negative to NHPB after 12 days, whereas 20% of shrimp fed on *Navicula* sp. were to NHPB positive. Regarding to Artemia, NHPB were detected in the organisms (dead or alive), four and eight days post inoculation. Microalgae and Artemia controls remained negative for NHPB during the experimental period. The results suggest that planktonic microalgae (*C. muelleri* and *Dunaliella* sp.) cannot be vectors of NHPB, while there is a possibility that benthic microalgae (*Navicula* sp.) and *Artemia* sp. can act as vectors of the bacteria.

1. Introduction

Necrotizing hepatopancreatitis bacteria (NHPB) is an intracellular Gram-negative polymorphic bacterium that affects penaeid shrimp (Frelier et al., 1992; Lightner and Redman, 1992; Lightner and Redman, 1994). In early 1990's, microscopical studies revealed the presence of different morphotypes of NHPB, localized exclusively in the cytoplasm and hepatopancreatic tubular epithelium of shrimp. Though morphologically they are markedly distinct, they represent different life stages of a complex organism (Frelier et al., 1992, Lightner and Redman, 1992). The bacterium is a member of the subclass α-Proteobacteria and it is considered as pathogen for shrimp; for instance, experiments where shrimp have been infected with NHPB reported that none of the organisms recovered from the disease (Loy and Frelier, 1996; Vincent and Lotz, 2005). Particularly, NHPB can cause near to 95% of losses in the production of farms (Frelier et al., 1992); in recent years mass die-offs have decreased due to implementation of sanitary protocols and the use of antibiotics. However, NHPB is still considered as a major problem for the shrimp farming industry in American countries.

The spread of NHPB toward farms from other countries threatens the development of shrimp aquaculture worldwide. In particular, severe epizooties of cultured *Litopenaeus vannamei* occurred in Mexico (Sonora and Sinaloa) during the last years and the mortality was attributed to NHPB infection (Ibarra-Gamez et al., 2007). In spite of that, information regarding to the life cycle and spread dynamics of NHPB is scarce. For instance, vectors of NHPB are still unknown; additionally, NHPB have been successfully transmitted and cultured only in shrimp hepatopancreas, which is the reason explaining such lack of information regarding the bacteria.

Shrimp aquaculture is frequently practiced at semi-intensive scale, which means that reared shrimp usually fed on a combination of formulated feed and natural food, such as phytoplankton and zooplankton (Martínez-Córdova et al., 2009). For instance, different microalgae and micro crustacean species are commonly present within shrimp culture systems. In addition, microalgae species such as *Chaetoceros muelleri*, *Dunaliella* sp. and *Navicula* sp., and branchiopod crustacean species such as *Artemia* sp. are almost universally used as food source during shrimp larviculture, pre-growout and growout phases (Becerra-Dorame et al., 2010; Campaña-Torres et al., 2010, Lopez-Elías et al., 2005; Pereira-Melo et al., 2010).

The presence of phytoplankton and zooplankton and their effect as disease vectors within aquatic ecosystems have been recognized (Drake et al., 2005; Sahul Hameed and Balasubramanian, 2000). In order to understand the spread dynamics of the bacteria and perform safety protocols, it becomes important to evaluate the phytoplankton and zooplankton organisms linked with the culture and life cycle of shrimp, as possible carriers of NHPB.

* Corresponding author. Tel.: +52 662 2892400.
E-mail address: tgollas@ciad.mx (T. Gollas-Galván).

0044-8486/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquaculture.2011.05.043

The aim of this study was to evaluate different microalgae species (*C. muelleri*, *Dunaliella* sp. and *Navicula* sp.) and Artemia (*Artemia franciscana*) as possible vectors of NHBP.

2. Materials and methods

The organisms evaluated as possible NHPB carriers were three species of microalgae, a diatom (*Chaetoceros muelleri*), a cloroficeae (*Dunaliella* sp.), a benthic (*Navicula* sp.) microalgae and the branchiopod crustacean, *A. franciscana*.

2.1. Microalgae experiment

2.1.1. Microalgae culture

Microalgae species were obtained from the microalgae culture laboratory at the Universidad de Sonora. They were scaled from 2 mL tubes to 2 L Erlenmeyer flasks, using f/2 medium ($1\ mL \cdot L^{-1}$) as nutrient source. Conditions of microalgae culture were: constant temperature (25 °C), aeration and illumination ($115\ \mu mol \cdot m^{-2}\ s^{-1}$). Six flasks per microalgae species were maintained.

2.1.2. Microalgae exposition to NHPB

The inoculum for microalgae consisted on shrimp hepatopancreas infected with NHPB (HP_{NHPB}), which was macerated, homogenized (Ultra-Turrax homogenizer, IKA Works Inc., USA) and dissolved in seawater filtered by 0.45 μM (1:1, w/v); thereafter, the inoculum was filtered through a screen (mesh 0.3 mm). Finally, inoculums were added to each flask at a rate of $0.5\ mL \cdot L^{-1}$ and mixed.

Prior to inoculation (control) and eight days post inoculation, microalgae samples were collected from each flask. To collect microalgae samples, flasks were mixed thoroughly and a volume of 100 mL was extracted. Samples were then filtered through GF/C Whatman filters in order to have microalgae concentrates. The microalgae concentrates were washed with 50 mL of sodium thiosulfate (0.3%) and two times with 50 mL of sterile marine water. Both, filtered water and microalgae concentrates were stored at −20 °C for further PCR analysis.

2.1.3. Shrimp inoculation

In order to verify if NHPB was still viable in microalgae flasks, additional samples (100 mL each) were extracted. The microalgae concentrates from each flask were pooled and resuspended in 3 mL on sterile marine water; suspensions of *C. muelleri*, *Dunaliella* sp. and *Navicula* sp. were used to inoculate adult white shrimp (*L. vannamei*, ~15–17 g). Twenty shrimp were inoculated per microalgae species and 100 μL of microalgae suspension were supplied by forced feeding (Gracia-Valenzuela et al., 2011). Twelve days post inoculation, shrimp were sacrificed and dissected to extract HP and store them at −20 °C. Other three groups of shrimp were fed with microalgae (*C. muelleri*, *Dunaliella* sp. and *Navicula* sp. respectively) free of NHPB. Shrimp used for the experiment were certified as free of NHPB by Centro de Investigaciones Biológicas del Noroeste.

2.2. Artemia experiment

Artemia cysts were decapsulated and haphazardly distributed into six experimental tanks (10 L) at a density of $3\ organisms \cdot mL^{-1}$. The organisms were fed on microalgae *Dunaliella* sp. during the first 20 days; after that, microalgae inputs were replaced by formulated feed Camaronina (Purina®, Hermosillo, Sonora, México) at a rate of 5–6% of total $biomass \cdot day^{-1}$. By the 30th day, Artemia were inoculated with NHBP. The inoculum consisted on HP_{NHPB} dissolved in glycerol (1:1, w/v), the solution of HP_{NHPB} + glycerol was combined with pulverized formulated feed, at a ratio of 1:1 (v/w). Thereafter, the inoculums (1 mL HP_{NHPB} + glycerol + formulated feed) were resuspended in 10 mL of sterile seawater and added into three experimental tanks, while the other three tanks received the same inoculums but without NHPB (1 mL HP + glycerol + formulated feed).

Artemia were sampled at days 4th and 8th of the trial and stored at −20 °C. Samples were analyzed at five different locations from each tank, each sub sample consisted of 10 organisms, *i.e.* 50 $Artemias \cdot tank^{-1}$. Sampled Artemia were placed on a screen (0.5 mm mesh) and thoroughly washed eight times with sterile marine water and two other with sterile distilled water; Artemia were observed through a microscopy (10× and 40×) to verify the absence of organic material attached to their bodies. Additionally, dead organisms and water samples were also collected.

2.3. NHPB detection

The presence of NHPB was confirmed in water, microalgae, shrimp and Artemia by molecular tools.

2.3.1. PCR tests

DNA was extracted from water, microalgae, shrimp HP and Artemia by a GENECLEAN SPIN kit (Qbiogene®). In particular, samples of HP and Artemia were thoroughly grinded and homogenized by a tissue homogenizer Ultra-Turrax T23 (IKA Works Inc., USA) prior to begin to the procedure of DNA extraction.

Specific primers were designed considering the sequence GenBank **U65509**: NHP/F2: 5′-CGT TGG AGG TTC GTC CTT CAG T-3′, NHP/R2: 5′-GCC ATG AGG ACC TGA CAT CAT C-3. A PCR kit was used to analyze the samples (Promega, Corp. USA) under the following conditions: 1 cycle at 95 °C – 5 min, 35 cycles 94 °C – 1 min, 60 °C – 1 min, 72 °C – 1 min, and one final cycle at 72 °C – 10 min.

PCR products were analyzed by electrophoresis using Agarose gels (2%; E-Gel, Invitrogen); the amplicons were observed by using an UV Transilluminator adapted to a specialized camera Gel-Logic 100 Imaging System (KODAK, Germany) and analyzed by the software Kodak Imaging System 4.0 (KODAK, Germany). The PCR products were purified using a PCR purification kit QIAquick® (QIAGEN, USA) following the manufacturer's specifications; thereafter, the purified samples were prepared and sent to a specialized laboratory (Centro de Investigaciones Sobre Enfermedades Infecciosas [CISEI]) to be sequenced. Nucleotide sequences were compared with the sequence U65509 GenBank in the algorithm Blast N of the National Center for Biotechnology Information Bethesda, MD (http://www.ncbi.nlm.nih.gov/BLAST/).

3. Results

3.1. Microalgae experiment

Necrotizing hepatopancreatitis bacteria was not detected by PCR in any of the microalgae concentrates or filtered water from each treatment prior to the inoculation (Fig. 1). After inoculation, NHPB was detected in filtrated water of each treatment and also in the benthic microalgae (*Navicula* sp.); however the bacteria were not detected in *C. muelleri* and *Dunaliella* sp. (Fig. 1). A 379-bp amplicon was observed in Agarose gel when a sample resulted positive (Table 1).

Shrimp fed with *C. muelleri* and *Dunaliella* sp. previously exposed to HP_{NHPB} resulted negative to NHPB at 12 days post inoculation; in contrast, 20% of shrimp fed on *Navicula* sp. resulted positive to NHPB; the bacteria were detected in one dead shrimp at 6th day post inoculation, while the rest were detected at 12th day. Shrimp fed on any of the three microalgae species considered as free on NHPB resulted negative (Table 1).

3.2. Artemia experiment

Necrotizing hepatopancreatitis bacteria were not detected in Artemia prior inoculation with HP_{NHPB}. Thereafter, NHPB detection resulted positive in 80% of Artemia samples at 4th day post