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# DEVELOPMENT OF A SIMPLE METHOD TO INOCULATE NECROTIZING HEPATOPANCREATITIS BACTERIUM IN *ARTEMIA* SP.

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*Abstract* - Different protocols were performed to evaluate their effectiveness to inoculate necrotizing hepatopancreatitis bacterium (NHPB) into *Artemia* sp. Protocol-A consisted of adding to water 0.2 $mL \cdot L^{-1}$ of bacterial inoculum composed of infected hepatopancreas ($HP_{NHPB}$) and glycerol (1:1 w/v). Protocol-B consisted of adding the same inoculum but at a ratio of 2 $mL \cdot L^{-1}$, whereas protocol C consisted of adding 0.2 $mL \cdot L^{-1}$, of $HP_{NHPB}$+glycerol+formulated feed (0.5:0.5:1 w/v/w). Controls for each treatment consisted of similar inoculums but using healthy hepatopancreas. Artemia from A and B treatments were negative for NHPB after four and eight days post-inoculation, whereas Artemia from treatment C resulted as positive to NHPB after four (75% of individuals) and eight (100%) days post-inoculation. The results suggest that the incorporation of formulated feed into the inoculums induced the bacteria's consumption of Artemia.

*Key words: Artemia* inoculation, NHP, bacterial inoculums.

## INTRODUCION

Branchiopod crustaceans have been reported as disease transmitters within aquatic ecosystems (Holdich and Pöckl 2007). In particular, *Artemia* sp. is widely used in the aquaculture of fish and crustaceans; additionally, they are commonly observed in diverse aquatic habitats, being a food source for diverse aquatic animals (Campaña-Torres et al., 2010). Thus, it is possible to hypothesize that *Artemia* could play a role as a transmitter of pathogenic organisms; in fact, some authors have demonstrated that *Artemia* is a vector for some virus and bacteria (Cano et al., 2009; Lone et al., 2009).

Several laboratory studies have been conducted to evaluate zooplankton species as possible disease vectors. However, it is difficult to perform certain laboratory experiments in order to know the role of *Artemia* or other small size organisms in the spread of pathogenic virus and bacteria because they cannot be inoculated by forced feeding or injection as can be done with larger animals. Thus, the few strategies to achieve such a goal include promoting the voluntary consumption of inoculums by *Artemia* and/or, inducing a physical contact among the pathogen organisms and *Artemia*.

Developing a simple but effective protocol to inoculate any bacteria into small size organisms such as *Artemia* could be a useful tool to study and understand the spread and life cycle of different bacteria related to *Artemia* and other small/micro crustaceans.

Necrotizing hepatopancreatitis bacterium (NHPB) was selected to evaluate different inocula-

tion procedures in *Artemia* sp. NHPB has caused the collapse of shrimp production in American and Latin American farms. The inoculation of NHPB into *Artemia* has been a difficult task in our laboratory. In addition, this bacterium has been successfully inoculated only into the penaeid shrimp (Gracia-Valenzuela et al., 2011). The aim of this experiment was to perform and evaluate different protocols to inoculate NHPB in *Artemia* sp.

## MATERIALS AND METHODS

The protocols used to inoculate NHPB into *Artemia* were based on modifying the composition and concentration of bacterial inoculums. Three different procedures were evaluated; the experimental design consisted of triplicate tanks per treatment group. Each tank was a plastic container with 10 L of sterile and filtered marine water (salinity 35‰, temperature 25°C, $NH_3$-$NH_4$ 0.01 mg·$L^{-1}$) and 2 *Artemia*·$mL^{-1}$ (35 day-old) were haphazardly stocked. *Artemia* were starved for 24 h prior to inoculation.

Necrotizing hepatopancreatitis bacteria was obtained from shrimp hepatopancreas sampled at commercial farms reporting the presence of the disease. The presence of the bacterium was confirmed by polymerase chain reaction and sequence analysis. Afterwards, the infected hepatopancreas were pooled and macerated to have homogeneous inoculums. The viability of the bacterium was confirmed by infecting healthy shrimp following the method described by Gracia-Valenzuela et al. (2011) and detecting the presence of NHPB after seven days.

In the first attempt (A), a NHPB inoculum was prepared, which consisted of macerated and homogenized shrimp hepatopancreas infected with NHPB ($HP_{NHPB}$), and mixed and homogenized with glycerol at a rate of 1:1 (w/v) (Ultra-Turrax T23; IKA Inc., USA). Afterwards, the inoculums were added and mixed into the water at a rate of 0.2 mL·$L^{-1}$.

The second attempt (B) was similar to the first procedure, but the inoculums were added at a rate 10 times higher (2 mL·$L^{-1}$). For the third attempt (C), the $HP_{NHPB}$+glycerol inoculum was mixed with formulated shrimp feed previously pulverized (Malta Cleyton®) at a ratio of 1:1 (v/w) and mixed into the water (0.2 mL·$L^{-1}$). Controls for each treatment entailed adding similar inoculums but HPs free of NHPB were used.

Samples of *Artemia* were collected at 4 and 8 days post-inoculation and washed thoroughly with sterile marine water and distilled water, using a screen (0.5 mm mesh). After washing, *Artemia* was observed through a microscope (at 40 x magnification) to discard the presence of bacterial aggregates on the body. *Artemia* sub-samples were collected at four different locations within each experimental tank (10 organisms·$location^{-1}$).

The presence of NHPB in *Artemia* was detected by the polymerase chain reaction. DNA was extracted using a commercial kit (GENECLEAN SPIN kit, Qbiogene®), and special primers were designed and used, considering the sequence U65509 GenBank: NHP/F2: 5'-CGT TGG AGG TTC GTC CTT CAG T-3', NHP/R2: 5'-GCC ATG AGG ACC TGA CAT CAT C-3. Finally, PCR (PCR kit, Promega, Corp. USA) was performed under the following conditions: one cycle at 95°C for 5 min, 35 cycles 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and one final cycle at 72°C for 10 min. Agarose gels (2%; E-Gel, Invitrogen Corp.) and UV transilluminator (Gel-Logic 100 Imaging System, KODAK) were further used to visualize the amplicons. The PCR products were purified using a purification kit (QIAquick®; QIAGEN, USA) and sent to a specialized laboratory (CISEI) to determine the nucleotide sequence. Thereafter, the sequences were compared to the sequence U65509 GenBank in the algorithm Blast N of the National Center for Biotechnology Information Bethesda, MD.

## RESULTS AND DISCUSSION

The first two protocols (A and B) did not succeed to inoculate NHPB into *Artemia*; the organisms sampled 4 and 8 days post-inoculation were negative for NHPB. In contrast, *Artemia* was positive for NHPB

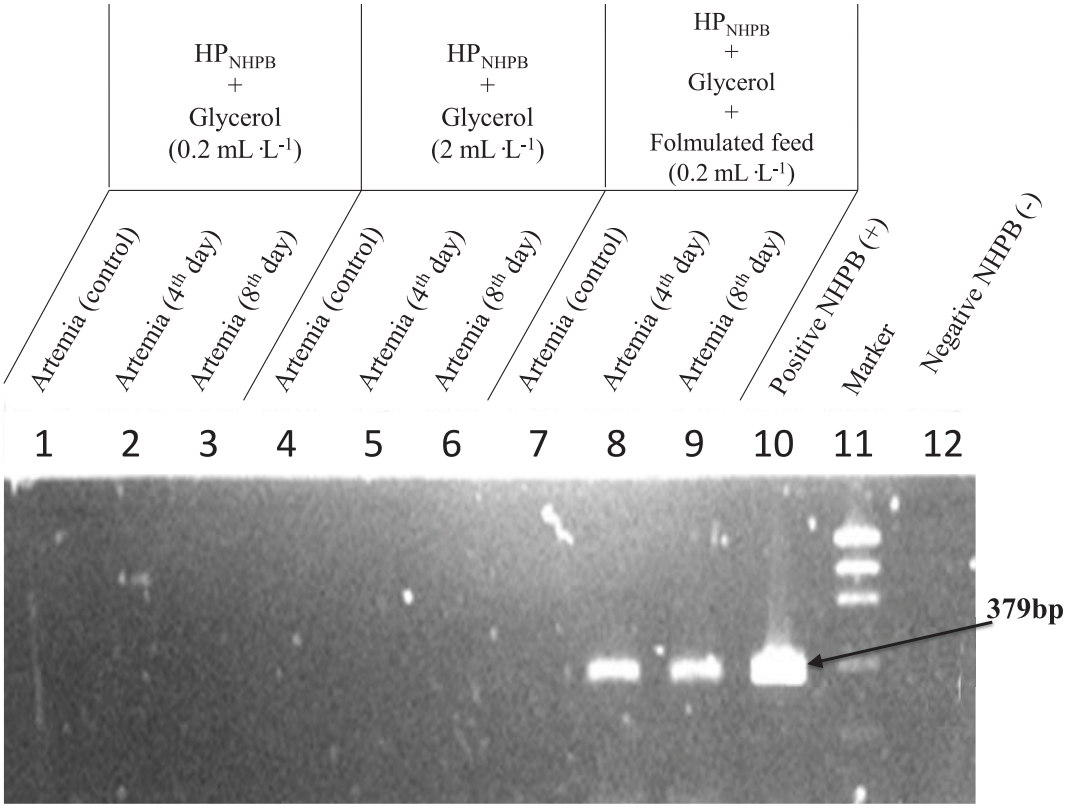


**Fig. 1.** Agarose gel electrophoresis analysis of PCR amplicons from extracted DNA from *Artemia* inoculated with NHPB by three different protocols. Protocol A: lanes 1-3, B: lanes 4-6, C: lanes 7-9. The positive NHPB control is shown in lane 10 (379-bp amplicon), the negative control in lane 12 and the low DNA mass ladder marker in lane 11 (Invitrogen). 75% and 100% of sampled *Artemia* were positive four and eight days respectively after inoculation in the attempt using formulated feed.

in the third protocol; amplicons of 379-bp were observed in *Artemia* sampled on 4th and 8th days (Fig. 1). The bacteria were detected in 75% of the organisms on 4th day, and in 100% on 8th day. The control treatments of all the experimental protocols remained negative for NHPB during the trial. The amplicons sequences matched 100% with the NHPB reference sequence of GenBank (U65509).

Despite *Artemia* being starved, apparently they did not consumed the inoculated HP; it is possible that glycerol affected their attraction to HP. However, we tested pure and macerated $HP_{NHPB}$ as inoculums, and the results were also negative (unpublished data). Thus, these results suggest that it is difficult, if not impossible, to transfer NHPB from $HP_{NHPB}$ to *Artemia*, probably because the *Artemia* were not stimulated to consume the inoculum. It is possible that some experiments failed to demonstrate that *Artemia* was a vector of certain bacteria, or failed in trying to enrich *Artemia* with probiotic bacteria, because the bacteria did not enter through their digestive tract. Moreover, the inoculation of *Artemia* or other zooplankton species is commonly carried out through the exposure of the organisms to bacteria strains added to the water (Marques et al., 2005). Such a protocol may be useful in some experiments but not in others.

Therefore, it is important in particular experiments to assure the consumption of inoculums by *Artemia* or the organism that is being evaluated. For instance, results from protocols A and B could conclude that *Artemia* cannot be inoculated by NHPB, but in protocol C the opposite was demonstrated.

NHPB was detected only after four days from inoculation by protocol C, which indicates a rapid bacterial proliferation when they are attached to a substrate (formulated feed) consumed by *Artemia*. Vincent and Lotz (2005) demonstrated that shrimp exposed to $HP_{NHPB}$ resulted positive after 6-21 days; they observed that the bacterial spread was induced by the consumption of $HP_{NHPB}$ by experimental shrimp. Thus, the reason of the fast proliferation of NHPB could be attributed to a rapid consumption of the inoculums. In addition, the rapid consumption of the inoculums could be associated to the feeding attractants contained in the artificial feed; for instance, the formulation and addition of feeding attractants in shrimp feed were performed considering the chemical communication process in crustaceans (Barki et al., 2011).

Future experiments could contemplate the incorporation of artificial feed or feeding attractants for crustaceans in their inoculum formulation as a strategy to assure the consumption of any bacteria by *Artemia* or other zooplankton species. Such a protocol may avoid erroneous results. Moreover, this simple method could be useful in *Artemia* enrichment practices with probiotic bacteria.

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