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Efficacy of phage therapy to prevent mortality during the vibriosis of brine shrimp

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ABSTRACT

Phage therapy is an alternative to control bacterial pathogens in aquaculture. The extensive use is apparently feasible, however it is necessary to know its potential limitations arising from the particular characteristics of the aquatic environment in terms of the dynamics of bacterial infections, in particular because under some circumstances, the direct release of phages is the unique alternative to apply the treatment to the cultures. In the present study the efficacy of phage therapy was evaluated under gnotobiotic conditions during the induced vibriosis of *Artemia franciscana*. Axenic brine shrimp nauplii were infected with *Vibrio parahaemolyticus* and treated with phage therapy, the effect of the reduction in the phage dose and the effect of delay treatment were evaluated. The vibriosis was successfully prevented by phage therapy; a single dosage of vpms1 phage was effective to eliminate the adverse effects of *V. parahaemolyticus* in brine shrimp ($P < 0.001$) and their efficacy was not affected by the reduction in the dosage, even at a multiplicity of infection of 0.45. However, its beneficial effects were compromised during the infection progress; when the application of phages was delayed, phage therapy was ineffective to control the mortality induced by *V. parahaemolyticus*. In conclusion, under the evaluated conditions the phage therapy was effective to prevent vibriosis in brine shrimp, however, in advanced infections their ability to control the vibriosis is limited.

1. Introduction

In recent years the use of antibiotics in aquaculture was restricted because of the worldwide increase of antibiotic-resistant bacteria (FAO/OIE/WHO, 2006). Phage therapy is an alternative to prevent and control bacterial infections; even under some circumstances it has already been proven to be medically superior to antibiotics.

During the last years, the phage therapy has been advertised as an alternative to control pathogenic bacteria in marine organisms (Almeida et al., 2009; Efrony et al., 2007; Karunasagar et al., 2005, 2007; Matsuzaki et al., 2005; Morrison and Rainnie, 2004; Ronda et al., 2003; Teplitski et al., 2009). In appearance phage therapy is an eco-friendly alternative to prevent or to control bacterial infections in aquaculture (Almeida et al., 2009; Shivu et al., 2007). The attributed advantages for phage treatment over chemotherapy include: 1) high specificity; that means no effect on beneficial bacteria and compatibility with the use of probiotics, bioremediation or biological filtration systems; 2) self-replication ability; 3) high stability in the environment and 4) not toxic or allergic effects.

The value of phage therapy to control of bacterial infections in fish was proved (Nakai et al., 1999; Park and Nakai, 2003; Park et al., 2000) and promising results were obtained in the control of shellfish pathogens (da Silva, 2005; Li et al., 1999; Nakai and Park, 2002; Pelon et al., 2005; Tai-wu, 2000; Vinod et al., 2006); however, the phage therapy is ineffective under some circumstances; for example, the phage therapy was recommended only as a preventive procedure for furunculosis in salmonids because it did not produce the expected beneficial effect during experimentally induced infections (Imbeault et al., 2006; Verner–Jeffreys et al., 2007).

In some cultures, for phage therapy, the phages cannot be supplied by oral, intraperitoneal, intramuscular or topical routes, for example in the larval cultures of fish and shellfish (where mass mortalities are associated to opportunistic bacteria) the use of phage therapy could imply the direct release of phages into the culture water. In consequence the released phages will cross some natural barriers to arrive to the specific site of infection (i.e. intestine) and their success could be affected by the environment, nature of infection or phage attributes. The effective, safe and controlled use of phages in aquaculture will require detailed information of the properties and behavior of specific phage/bacterium systems, including phage-kinetics information i.e. the time of permanence in the water or in the cultured organisms and their impact on the microbial community, considering that phages interact, replicate, evolve, and exhibit singularities unknown in the kinetics of conventional drugs (Payne and Jensen, 2003).

The aim of the present study was to test the efficacy of phage therapy during brine shrimp experimental infection with their pathogen *Vibrio parahaemolyticus*. This bacterium was selected as model because it can infect a number of organisms, including humans, has been reported as dominant in the shrimps and brine

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shrimp microbiota and can cause intestinal lesions and mortality in crustaceans in few hours (Martin et al., 2004).

2. Material and methods

2.1. Bacteria

V. parahaemolyticus, strain 17802 was directly obtained from the American Type Culture Collection (ATCC) and was cultured in marine agar (MA) (Yeast Extract 1 g, Bacto® Peptone 5 g, Bacto® Agar 17 g and aged marine sea water 1 L) at 30 °C. For experiments the cells were harvested at 24 h and adjusted at an optic density of 1 at 585 nm ($OD_{585} = 1$) (corresponding at ca. 10^8 CFU ml^{-1}) in artificial sea water (ASW) (Instant Ocean®).

2.2. Phage isolation and culture

The phages used in this investigation were isolated during an extensive screening program which begins in 2003 in the Northwest coast of México. The phage Vpms1 is a double stranded DNA podovirus, was selected from our collection by their strong lytic effect on *V. parahaemolyticus* (ATCC 17802) and because their complete genome was recently obtained (sequence deposited in NCBI GenBank with accession number JX880072). This phage was originally isolated from commercial samples of the clam *Megapitaria squalida* collected from La Paz bay, B.C.S. México. The isolation was achieved following the standard enrichment and filtration procedures described by Carlson (2005). Briefly, the digestive glands of five clams were separately homogenized in 200 ml of sterile ASW and inoculated with 1 ml of *V. parahaemolyticus* suspension in sterile ASW ($OD_{585} = 1$). The homogenates were incubated by 24 h at 30 °C and 10 ml aliquots were filtered using 0.2 μm (PALL Acrodisc). The presence of phages in the filtrates was corroborated by plaque formation on a lawn of *V. parahaemolyticus* prepared on Marine Agar 2216 (Difco).

Positive supernatants were serially diluted and inoculated in combination with *V. parahaemolyticus* in order to promote the formation of single plaques. Individual plaques were transferred to 10 ml of Marine Broth previously inoculated with *V. parahaemolyticus* and incubated at 30 °C during 24 h and then 0.2 μm filtered. A clone was purified by serial isolations of plaques. The filtrates with phages were stored at −70 °C.

For massive production, the phages were inoculated in 500 ml of marine broth previously inoculated with the target bacterium. After 24 h at 30 °C the cultures were 0.2 μm filtered and maintained at 4 °C until their use. The titer in the stock used in the experiments was determinates in $2.7 \cdot 10^9$ PFU $\cdot$ ml^{-1} according to Carlson (2005).

2.3. Inducing the V. parahaemolyticus infection in brine shrimp nauplii

2.3.1. Germfree brine shrimp cultures

Bacteria free cultures of brine shrimp were obtained by a procedure previously established in our laboratory. Briefly, under sterile conditions, 0.5 g of *Artemia franciscana* cysts (INVE) were hydrated in sterile distilled water (SDW) and decapsulated during 30 s in a solution 1:1 of commercial hypochlorite (Cloralex®); washed thoroughly with sterile sea water and disinfected during 15 s in 1% benzalkonium chloride. The cyst were washed again, transferred to 2 L flasks with 700 ml of sterile sea water and maintained at 28 °C under continuous illumination and 0.2 μm filtered aeration. At 19 h, the nauplii were harvested under aseptic conditions and transferred to sterile experimental culture vessels with 100 ml sea water at a density of 100 nauplii per vessel. The bacteria-free condition was corroborated by viable counts in 2216 medium (Difco) and by direct microscopic observations in concentrated and stained samples (Gram and Acridine-Orange stains) under a light-epifluorescence microscope (Olympus® BX60). If any contamination was detected, the results of the experimental run were not accepted.

Hereafter, each vessel with 100 nauplii will be referred as experimental unit.

2.3.2. Experimental infection

The dose and time of infection with *V. parahaemolyticus* strain ATCC17802 were adjusted to reach the 50% mortality (LD_{50}) at approximately 48 h.

2.3.2.1. Dose. Bacteria-free nauplii (obtained as previously described) were infected with different dosages of *Vibrio parahaemolyticus*. Overnight cultures of ATCC 17802 were harvested and the optic density adjusted at $OD_{585} = 1$. Experimental units were inoculated in triplicates with 100, 200, 300, 400, 500, 600, 700 and 800 μL of the bacterial suspension ($OD_{585} = 1$) and three uninfected units were used as controls. The experimental units were maintained at 28 °C and the brine shrimp survival was recorded at 72 h post infection (PI). This experiment was repeated three times in order to evaluate their precision. The dosage of 50% mortality (LD_{50}) was estimated using a probit approach.

2.3.2.2. Kinetics of mortality during the V. parahaemolyticus infection. Twenty eight experimental units (containing bacteria-free nauplii) were inoculated with a single dosage of *V. parahaemolyticus* (600 μL) as previously described and were incubated at 28 °C, the mortality was recorded at 0, 8, 16, 24, 32, 40 and 48 h. At each time, a group of four experimental units were randomly selected and the numbers of live organisms were counted under a stereomicroscope.

2.4. Phage therapy during the V. parahaemolyticus infection

The efficacy of phage treatment to prevent the mortalities in experimentally infected nauplii was achieved under three different conditions: 1) single high dosage at early stages of infection; 2) reduced dosages of phages (to promote an active therapy) and 3) during different degrees of progress of the infection.

2.4.1. Single dosage effect

Ten experimental units (containing bacteria-free nauplii) were infected with 600 μL *V. parahaemolyticus* (at $OD_{585} = 1$); five of them were randomly selected and treated with 1 ml of the phage stock suspension (to reach a multiplicity of infection MOI of 45); and five untreated experimental units serve as controls. Also five experimental units without bacteria or phages (axenic) were the blanks of this experiment. The experimental units were incubated at 28 °C and the mortality was recorded at 48 h.

2.4.2. Effect of reducing the dose

Experimental units with infected brine shrimp nauplii (as previously described) were treated with a single dose of 1000, 100, 50 or 10 μL of Vpms1 phage stock (at $2.7 \cdot 10^9$ PFU ml^{-1}), to reach 45, 4.5, 2.25 and 0.45 MOI respectively. The evaluation was achieved by triplicate and the experimental units were incubated at 28 °C. Mortality was recorded at 48 h. Axenic controls and controls without phage treatment were included in this experiment.

2.4.3. Effect of delayed treatment

The efficacy of Vpms1 to control the brine shrimp vibriosis at different degrees of progress of infection was evaluated under previously described conditions. Fifty experimental units with bacteria-free nauplii were simultaneously infected with *V. parahaemolyticus* as previously described (600 μL, $OD_{585} = 1$) and were randomly assigned in groups of five for each time of phage treatment. A single 10 μL dose of Vpms1 stock (at $2.7 \cdot 10^9$ PFU ml^{-1}) was applied at 0, 3, 10, 20 and