Aquaculture 434 (2014) 208–211

Contents lists available at ScienceDirect

Aquaculture

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

Phage therapy against *Vibrio parahaemolyticus* infection in the whiteleg shrimp (*Litopenaeus vannamei*) larvae

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ARTICLE INFO

Article history:
Received 14 July 2014
Received in revised form 8 August 2014
Accepted 12 August 2014
Available online 19 August 2014

Keywords:
Phage therapy
Litopenaeus vannamei
Vibrio parahaemolyticus
Shrimp larvae

ABSTRACT

Vibrio parahaemolyticus is an important cause of disease, mortality, and economical losses in the shrimp aquaculture industry. Bacteriophages are natural bio-controlling agents, broadly recognized for their ability to reduce pathogen populations. Hence, in the present study, we evaluated the effectiveness of phage therapy in the prevention and control of vibriosis in *Litopenaeus vannamei*. Vibriosis was induced in shrimp larvae with 2×10^6 CFU mL^{-1} of *V. parahaemolyticus*. The infected larvae were treated with different doses of selected phages and their efficacy was evaluated at different times after their application. Results revealed that selected lytic phages (A3S and Vpms1) are effective to reduce mortality caused by *V. parahaemolyticus*. In both cases, the early application (at 6 h post-infection) was effective to avoid mortality. Low multiplicity of infection (MOI) values (<0.1) were enough to counteract *V. parahaemolyticus* infection. Delayed phage applications (>6 h post-infection) hindered mortality and the progress of infection. This study provides the basis for the use of bacteriophages in the prevention and control of *V. parahaemolyticus* in shrimps.

1. Introduction

Vibrio spp. are the putative cause of strong economic losses in the shrimp industry; they can infect all life stages (from eggs to broodstock); generating in most cases 100% mortality (Prayitno and Latchford, 1995; Harris and Owens, 1999; Aguirre-Guzmán et al., 2010).

Vibrio parahaemolyticus (VP) is a Gram-negative bacterium that has been commonly associated with infections in aquatic organisms. In addition, it is a major concern for human health because it is a leading cause of seafood-borne bacterial gastroenteritis worldwide (DePaola et al., 2003; Gopal et al., 2005; Zimmerman et al., 2007; Turner et al., 2013). In Mexican shrimp hatcheries, the presence of VP is monitored frequently and has been associated with necrosis, slow growth, muscle opacity, anorexia, and mortality during seed production (Balcázar et al., 2007; Aguirre-Guzmán et al., 2010). During 2013, some strains of VP were reported as the etiological agent of the acute hepatopancreatic necrosis syndrome (AHPNS/ESM) that caused the collapse of the shrimp aquaculture in Asia (Tran et al., 2013) and Mexico.

Currently there are scarce alternatives to control vibriosis during shrimp rearing, including some disinfectants and few legally allowed antibiotics (Santiago et al., 2009; Labreuche et al., 2012). However, new promising approaches are under development and, apparently, some of them can provide an acceptable level of control of pathogenic vibrios with little or null environmental damage.

The potential of phage therapy (use of bacteriophages to control bacterial infections) in aquaculture is gaining the interest of the scientific community and, during the last 5 years, different opinions and multiple reviews have been published. However, few efforts have been made to validate their efficacy or the possible impacts associated with the massive releasing of phages to the environment.

The early evaluations of phage therapy to prevent vibriosis produced very encouraging results; for example, during experimental shrimp larval production, Vinod et al. (2006) demonstrated that the application of *Vibrio harveyi* phages improves survival rate, even when compared with antibiotic-treated organisms. However, we can expect that, under commercial conditions, their actual effectiveness or apparent beneficial effect will be linked to the presence of specific pathogens, because phages have a narrow hosts range, and their ability to control ongoing infections is still unknown. Therefore, at this time, it is crucial to generate models to assess the conditions under which the phage therapy is effective and the factors that affect their efficacy. In the present study, we evaluated phage therapy as an alternative to prevent and control the damage produced by VP in the whiteleg shrimp larvae.

2. Methods

2.1. Bacteria and phages

V. parahaemolyticus ATCC 17802 was obtained directly from the American Type Culture Collection (ATCC). The stocks were maintained at −50 °C with 50% glycerol. For experiments, bacteria were cultured in marine agar (MA) and the cells were harvested at 24 h and adjusted

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http://dx.doi.org/10.1016/j.aquaculture.2014.08.018
0044-8486/

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®).The phages A3S and Vpms1 used in this study were previously isolated from healthy shrimp cultures (Makarov, 2008) and clams (Martínez-Díaz and Hipólito-Morales, 2013), respectively. Phage stocks were produced at 30 °C in fresh VP cultures; the lysates were centrifuged at 3500 rpm to eliminate bacterial debris and then filtered through 0.02-μm membranes. The number of viable phages was quantified by standard PFU counts and stored at 4 °C until use.

2.2. Shrimp larvae

Shrimp larvae at nauplius III stage were obtained from two commercial hatcheries (Acuacultura Mahr SA de CV, La Paz, BCS, Mexico, and BIOGEMAR SA, Salinas, Ecuador). For each experiment, a group of apparently healthy larvae (obtained from the collective spawn of at least 10 females) were transported to the laboratory and maintained in the carrying boxes until reaching the nauplius IV-V stage (N IV-V); then they were disinfected with 0.3 ppm chlorine dioxide (ClO_2) during 5 min, washed with sterile sea water and distributed in sterile containers with 100 mL artificial seawater (ASW) (Instant Ocean at 35 ppt) at a density of 1 larva mL^{-1}. At 24 h after disinfection, different treatments were applied (including infection or phage therapy) and maintained during 96 h at 30 °C. A gnotobiotic culture of *Chaetoceros calcitrans* was provided as the sole food source during experiments: at an initial dose of 1×10^4 cell mL^{-1} and, successively, adjusting to 1×10^5 cell mL^{-1}.

2.3. V. parahamolyticus challenge and phage therapy

Groups of 100 larvae (previously disinfected with ClO_2 and acclimatized as described in Section 2.2) were infected in the same container with a single dose of VP at 2×10^6 CFU mL^{-1} and treated with 100 μL of Vpms1 or A3S phage suspensions. Groups of VP-infected larvae that were no treated with phages were used as positive controls and uninfected larvae were used as negative controls or blanks. Each treatment was assayed in triplicate and the survival rate and vibriosis signs were recorded at 96 hpi (hours post infection).

2.4. Minimal effective dose of phages to control VP effects

Eighteen groups of 100 larvae at 24 h (previously disinfected with ClO_2 and acclimatized as described in Section 2.2) were infected with VP (at a 2×10^6 CFU mL^{-1} dose). The containers were randomly selected in groups of three and treated with different volumes of A3S or Vpms1 phages, to reach MOI values of 0.1, 1, and 10. Positive and negative controls (as previously described) were simultaneously maintained and all groups were analyzed at 96 hpi.

2.5. Effect of delayed application of phage therapy

Twenty-four groups of 100 larvae (previously disinfected with ClO_2 and acclimatized as described in Section 2.2) were simultaneously infected with VP (at a 2×10^6 CFU mL^{-1} dose), and triplicate groups were randomly selected to be treated with phages at different times (0, 6, 12, 24, and 36 hpi). Treatments comprised a single dose of 100 μL of Vpms1 or A3S phage suspension (reaching MOI values of 1 and 2, respectively). Positive and negative controls (as previously described) were maintained simultaneously and all groups were analyzed at 96 hpi.

2.6. Statistical analysis

Normality and homoscedasticity were evaluated using the Kolmogorov–Smirnov and Bartlett test, respectively, according to Zar (1999); data were analyzed by one-way ANOVA and Tukey multiple comparisons using Statistica 8.0 software.

3. Results

3.1. V. parahamolyticus challenge and phage therapy

VP infected larvae developed the typical signs of vibriosis, including empty digestive tract, red chromatophores, appendage deformations, and lethargy; mortality and changes in behavior were apparent at 48 hpi, and survival was significantly reduced as a result of infection ($p < 0.05$). The uninfected group had an average survival of 90 ± 6.8%, and no signs of vibriosis were recorded. In the VP-infected groups and treated with phages, most larvae appeared very active. The digestive tract was filled with food, few larvae showed signs of vibriosis, including loss of spines from the anterior appendages, and survival was not significantly reduced as compared to uninfected controls ($p > 0.05$) (Table 1).

3.2. Minimal effective dose of phages to control VP effects

The beneficial effects observed during the application of Vpms1 or A3S phages were not affected by the reduction in doses (Table 2). Doses corresponding to 0.1 MOI were effective to control the adverse effects caused by VP in shrimp larvae. The survivals recorded in shrimps treated with different doses of Vpms1 were not statistically different among them or compared with uninfected controls ($p > 0.05$). The highest survival rate (75 ± 4.5%) was recorded in organisms treated with A3S at 0.1 MOI; however, no significant differences were recorded between 0.1 and 1 MOI of A3S or when compared to uninfected organisms ($p > 0.05$).

3.3. Effect of delayed application of phage therapy

A single dose of Vpms1 or A3S, applied at any time, was enough to control the infection and mortality in shrimp larvae challenged with VP. Compared with uninfected controls, no significant reduction in survival was recorded in the groups challenged with VP and treated with Vpms1 at 0 to 12 hpi ($p > 0.05$), although minor signs of vibriosis were recorded. When Vpms1 phages were applied at 24 hpi or later, the survival was reduced; however, the effect caused by VP was lower than that recorded in challenged larvae without phage treatment (Fig. 1a). Larvae treated with A3S phage between 0 and 6 hpi did not show negative effects, and mortality was not significantly different from that of the control group ($p > 0.05$). Whenever A3S phage inoculation was delayed more than 6 hpi, survival rate decreased with time; however all treated groups showed significant differences ($p < 0.05$) as compared to larvae without phage treatment (Fig. 1b). In all cases, signs of vibriosis were apparently reduced by the application of A3S.

4. Discussion

Infections caused by *Vibrio* spp. are responsible for mass mortality in shrimp aquaculture and exert strong impacts on the economy of producing countries. In the past, their control was achieved mainly through antibiotic therapy, but the restriction in the use of antibiotics in aquaculture left a gap that needs to be compensated with new

Table 1
Effect of phage therapy on survival of whiteleg *L. vannamei* shrimp larvae during a *V. parahaemolyticus* (VP) challenge. (Different letters mean significant differences at $P < 0.05$).

Treatment	Survival (%) ± SD
Axenic control	87 ± 6.8^a
VP	59 ± 4.9^b
VP + A3S phage	80 ± 3.5^a
VP + Vpms1 phage	77 ± 3.0^a