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Efficiency of phage cocktails in the inactivation of *Vibrio* in aquaculture

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ABSTRACT

Aquaculture is one of the fastest growing sectors in the world, having a very important role in the economy. However, the losses associated with bacterial infections such as vibriosis, lead to huge economic costs. The regular use of antibiotics in aquaculture has resulted in the development of resistant strains, which have contributed to the inefficacy of antibiotics. To reduce the risk of the development and spreading of microbial resistance and to control the fish diseases in aquaculture, alternative strategies must be developed. Phage therapy can be an eco-friendly alternative to prevent and control pathogenic bacteria in aquaculture. However, phage bacterial resistance is already well documented but the use of phage cocktails can overcome this drawback. The aim of this study was to evaluate the efficiency of cocktails of two and three phages of *Vibrio parahaemolyticus* (VP-1, VP-2 and VP-3) to control *Vibrio* in aquaculture. All phages were effective against *V. parahaemolyticus*, however, the VP-3 phage was the most efficient one (additional reduction of more 2 log when compared with the other two phages). The use of cocktails with two and three phages was significantly more effective (reductions of 4 log after 2 h) than the use of VP-1 and VP-2 phages alone (reductions of 0.8 log after 2 h), however, the efficiency of VP-3 phage was similar for the phage alone and for the phage in the cocktails (reduction of 3.8 log and 4.2 log for VP-3 phage alone and in cocktails, respectively, after 8 and 6 h). All phages remained viable for a long time (at least 5–7 months) in marine water. The VP-3 phage presented a larger burst size and a shorter latent period (42 and 40 min, respectively) than the other two phages (9 and 120 min and 15 and 90 min, respectively, for VP-1 and VP-2 phages). Overall, the use of phage cocktails of two or three phages increased the efficiency of phage therapy against *Vibrio* (more efficient and faster bacterial inactivation), delaying the development of resistance by the bacteria and the use of *Vibrio* phages with high burst sizes and short lytic cycles also increases the efficiency of phage therapy.

1. Introduction

In commercial aquaculture, unfavorable conditions such as overfeeding, high temperature, fast growth, infrequent water renewal rate and improper removal of wounded and dead fishes from the farming area create favorable conditions for the emergence of bacterial diseases (Almeida et al., 2009). Bacterial infections, including multidrug-resistant strains, have been recognized as an important limitation to the development of the aquaculture production (FAO, 2009; Oliveira et al., 2012). Vibriosis, caused by the bacteria from the family Vibrionaceae, is currently responsible for most outbreaks in aquaculture. Vibriosis is caused by species of *Vibrio* (namely *V. parahaemolyticus*, *V. anguillarum*, *V. vulnificus*, *V. alginolyticus* and *V. salmonicida*) (Benediktsdottir et al., 1998; Hanna et al., 1992; Silva-Aciares et al., 2013; Sung et al., 1999; Almeida et al., 2009; Hanna et al.;, 1992; Noya et al., 1995; Sung et al., 1999; Toranzo et al., 1991).

V. parahaemolyticus is an important human bacterial pathogen that is widely distributed in marine environments, frequently isolated from a variety of seafood including codfish, sardine, mackerel, flounder, clam, octopus, shrimp, crab, lobster, crawfish, scallop and oyster (Liston, 1990; Su and Liu, 2007). This bacterium of marine environments is frequently associated with the development of acute gastroenteritis in humans by consumption of raw or undercooked contaminated seafood, particularly shellfish (Kaysner and DePaola, 2000). The regular use of artificial food supplemented with antibiotics in intensive and semi-intensive aquaculture systems, to prevent the spread of diseases and their massive use to control infections, has resulted in the development of resistant strains, which have contributed to the inefficacy of antibiotic treatments (Martinez, 2003). To reduce the risk of the development and spreading of microbial resistance and to control the fish diseases in aquaculture, alternative strategies must be developed (Defoirdt et al., 2011). Phage therapy can be used as an alternative to prevent and control pathogenic bacteria in aquaculture.

A major concern regarding the use of phages in the treatment of infectious diseases is the emergence of phage-resistant mutants (Gill and Hyman, 2010; Smith and Huggins, 1983). Resistance may arise due to the alteration or loss of the bacterial cell surface receptors, blocking of the receptors by the bacterial extracellular matrix, inhibition of phage DNA penetration, production of modified restriction endonucleases that degrade the phage DNA, or due to the inhibition of the phage intracellular development (Labrie et al., 2010). Mutations affecting phage

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receptors represent the most frequent cause of bacterial phage resistance (Heller, 1992; Labrie et al., 2010).

Although the development of phage-resistance, when only one phage is used, has already been reported (Levin and Bull, 2004; Merril et al., 2006; Nakai et al., 2010; Sandeep, 2006; Scott et al., 2007; Silva et al., 2013; Skurnik and Strauch, 2006; Tanji et al., 2005; Vieira et al., 2012), this limitation can be overcame by the combined use of more than one phage at the same time, that is, by the use of phage cocktails (Chan et al., 2013; Crothers-Stomps et al., 2010). Furthermore, previous reports suggested that virulent bacteria that are resistant to phage infection could be less fit or could lose their pathogenic properties (Anonymous., 1983; Capparelli et al., 2010; Filippov et al., 2011). Bacterial cell surface components that act as receptors for phage adsorption can also act as virulent factors, which may undertake mutation when bacteria develop resistance to phages, rendering them not pathogenic.

Phage cocktails not only potentially provide a means to circumvent resistance to the presence of a single phage but they also allow the treatment of multiple pathogens simultaneously (Cairns et al., 2009; Kunisaki and Tanji, 2010; Merabishvili et al., 2009). Therefore, the high specificity of bacteriophages, that sometimes can be considered to be a disadvantage of phage therapy, namely when the photogenic bacteria are not known, may be circumvented by the use of phage cocktails, which broaden the spectrum of action (Chan et al., 2013; Sulakvelidze et al., 2001).

The aim of the present study was to test the efficacy of phage cocktails of two and three *V. parahaemolyticus* phages (VP-1, VP-2 and VP-3) to control *V. parahaemolyticus* in aquaculture systems. The phages were tested alone and combined as cocktails. As the selection of appropriate bacteriophages is a key factor in the success of phage mediated-control of aquaculture infections, the three phages were characterized in terms of survival in the marine environment, host range, latent period and burst size.

2. Material and methods

2.1. Bacterial strains and growth conditions

The bacterial strains, *V. parahaemolyticus*, *V. anguillarum*, *Aeromonas salmonicida*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas segetis* and *Pseudomonas gingeri*, used in this study were previously isolated in our laboratory (Pereira, 2009; Pereira et al., 2011; Louvado et al., 2012). The other bacterial strains, *Photobacterium damselae* subsp. *damselae* (ATCC 33539), *Escherichia coli* (ATCC 13706), *Vibrio fischeri* (ATCC 49387), *Aeromonas hydrophila* (ATCC 7966), were purchased from ATCC collection. Fresh plate bacterial cultures were maintained in solid Tryptic Soy Agar medium (TSA; Merck, Darmstadt, Germany) at 4 °C. Before each assay, one isolated colony was aseptically transferred to 10 mL of Tryptic Soy Broth medium (TSB; Merck, Darmstadt, Germany) and was grown overnight at 25 °C. An aliquot of this culture (100 μL) was aseptically transferred to 10 mL of fresh TSB medium and grown overnight at 25 °C to reach an optical density (O.D. 600) of 0.8, corresponding to about 10^9 cells per mL.

2.2. Phage isolation and purification

Three phages (VP-1, VP-2 and VP-3) were isolated from marine water samples (salinity 18–21; pH 7.6–7.7) of a semi-intensive aquaculture (earthen pond aquaculture system Corte das Freiras located in the estuarine system Ria de Aveiro, latitude: 40°37′51.44″N, longitude 8°40′31.75″W, on the north-western coast of Portugal) using *V. parahaemolyticus* as host. Five hundred milliliters of water were filtered sequentially by 3 μm and then by 0.45 μm pore-size polycarbonate membranes (Millipore, Billerica, USA). Filtered water was added to 500 mL of TSB with double concentration and to 1 mL of bacterial culture. The mixture was incubated at 25 °C for 18 h at 80 rpm, and then filtered through a 0.45 μm membrane. The presence/absence of the bacteriophage was verified through the spot test (Vieira et al., 2012). Thirty microliters of the resulting filtrate were inoculated into TSA growth medium previously inoculated with the bacterial culture. The plates were incubated at 37 °C for 4–12 h and inspected for zones of clearing. Three successive single-plaque isolations were performed to obtain a pure phage stock. All lysates were centrifuged at 10,000 *g* for 10 min at 4 °C, to remove intact bacteria and bacterial debris. The phage stocks were stored at 4 °C and 1% chloroform (Scharlau, Spain) was added. The phage suspension titer was determined by the double-layer agar method using TSA as culture medium (Adams, 1959). The plates were incubated at 37 °C for 4–8 h and the number of plaques was counted. The results were expressed as plaque forming units per milliliter (PFU mL^{-1}).

2.3. Host range determination and efficiency of phage infection

The spot test was performed with the twelve bacteria to assess the bacterial susceptibility to the bacteriophage (Vieira et al., 2012). The plates were incubated at 37 °C for 4–12 h. The efficiency of plating was determined for the bacteria with positive spot tests (occurrence of a lysis zone) by the double-layer agar method using TSA as culture medium. Efficacy of plating for each host was calculated by comparison with an efficacy of 100% for the *V. parahaemolyticus* bacterium. For each phage, three independent experiments were done and the results were presented as the average of the three assays.

2.4. Phage survival determination

The survival of *V. parahaemolyticus* phages was tested in marine water of the aquaculture system Corte das Freiras, in three different dates, between May and July 2013. In each date, 50 mL of water were filtered through 0.45 μm and then by 0.22 μm pore-size membranes (Poretics, USA) which was followed by the addition of phage suspensions of about 10^7 PFU mL^{-1}. The samples were then incubated at 25 °C without shaking, in the dark. Phage titer was determined at time zero and at intervals of 12 h until the first day, 24 h until the fifth day, 48 h until the ninth day, 72 h until the twelfth day, 120 h until the forty-fifth day and 240 h until the end of the experiment (185 days), by the double-layer agar method. For each phage, three independent experiments were done.

2.5. One step growth assays

Mid-exponential host bacterial cultures of *V. parahaemolyticus* were adjusted to a 1 O.D. at 600 nm (corresponding to a cell density of 10^9 CFU mL^{-1}). Ten microliters of the phage suspension was added to 10 mL of the bacterial culture in order to have a multiplicity of infection (MOI) of 0.001. The phage was allowed to adsorb for 5 min at 25 °C, without shaking. The mixture was centrifuged at 13,000 rpm for 5 min, the pellet was re-suspended in 10 mL of TSB at 37 °C and was then serially diluted to 10–4. Samples (1 mL) were taken at 10–20 min intervals and subjected to phage titration by the double-layer agar method. Three independent assays were done.

2.6. Phage therapy assays

Phage therapy was performed using one phage alone (VP-1, VP-2 or VP-3) and with phage cocktails (two or all the three phages mixed together, each phage at the same concentration) using the bacterium *V. parahaemolyticus* as host, at a MOI of 100. The assays with two phages were performed with the following phage combinations: VP-1/VP-2, VP-1/VP-3, VP-2/VP-3 and VP-1/VP-2/VP-3 phages. In order to obtain a MOI of 100, 20 μL of the overnight *V. parahaemolyticus* culture (10^5 CFU mL^{-1}) and 300 μL of the phage suspension (10^7 PFU mL^{-1}) were inoculated into sterilized glass Erlenmeyers with 30 mL of TSB medium and incubated at 25 °C without agitation in the dark (test