Bacteriophages as biocontrol agents in aquaculture

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Aquaculture production (inland and marine) has been increasing globally reaching 80.1 million metric tons in 2016. Simultaneously the utilisation of fish food per capita has also been risen reaching 20.0 kg per year in 2016. However, the growing industry also experiences problems including diseases caused by viruses, bacteria, fungi, protozoans, helminths and parasitic crustaceans on valuable seafood products resulting in economic losses. Antimicrobial agents and chemical control strategies used to control such diseases are creating environmentally detrimental effects as well as encouraging development and dissemination of antibiotic resistant bacteria. Vaccine developments are costly and lengthy with application difficulties in farm settings. Accordingly, alternative therapies for controlling bacterial pathogens in aquaculture are gaining importance. One such measure is to use bacteriophages that are specific to disease causing bacteria.

Vibrio species are the main pathogens responsible for disease outbreaks which can result in 98.5–100% of mortality of the host animal within 72–96 h causing huge economic losses to hatcheries[1]. Examples include *V. tubiashii* infections that caused mortalities of oyster larvae in North America from 2006 to 2008 resulting in a decline of 59% in production at that time[2]. Moreover, antibiotic resistant species of *Vibrio* have also been on the increase. Mass mortality in the larvae of black tiger shrimp (*Penaeus monodon*) was reported to be caused by multi-drug resistant *V. harveyi* strains with resistance to cotrimoxazole, chloramphenicol, erythromycin and streptomycin[3]. Resistance of 15 *V. alginolyticus* isolates from oysters farmed in Korea against 16 different antibiotics including ampicillin, vancomycin and erythromycin has also been reported[4]. *Vibrio* spp. isolated from fish pond facilities in Nigeria were also reported to be resistant to tetracyclineoxazole (100%), oxytetracycline (99.4%) and chloramphenicol (73.1%)[5].

Aeromonas has been another pathogenic genus causing significant economic losses for aquaculture operations. Antibiotics again are extensively used to control diseases caused by the pathogenic species of this genus: examples include amoxicillin, ampicillin, cephamycin, cotrim and kanamycin[6,7]. However, according to the results from an antimicrobial susceptibility survey taken between 2013 and 2014, the sensitivity of these pathogens against the above-listed antibiotics decreased over this time thus devaluing the efficiency of antibiotic treatment. Highly virulent and antibiotic resistant strains to co-trimoxazole, tetracycline, florfenicol, ampicillin, trimethoprim/sulfamethoxazole, nalidixic acid, chloramphenicol, and nitrofurantoin were also reported[7–9]. Strains with complete resistance to methicillin, rifampicin, bacitracin and novobiocin were also reported for the same pathogen isolated from fish and prawns in South India[9].

Fish *nocardiosis* caused by *Nocardia* species in particular by *N. seriolae* is also on the increase in the South East Asia Pacific region. Erythromycin, oxolinic acid and fosfomycin resistant strains of the pathogen have also been reported[10].

While control of bacterial diseases has been attempted via different strategies during farming, after harvest unhygienic practices also constitute serious public health risk issues. Cross-contamination with pathogenic bacteria (e.g. *Escherichia coli*, *Campylobacter* and *Salmonella* spp) is one of the main causes of food poisoning after harvest. These pathogens can easily be spread to ready-to-eat foods, such as raw oysters and salads, through handling and

contaminated equipment or surfaces. In particular, during shucking of oysters, a significant risk of cross contamination can occur due to poor hygiene leading to gastrointestinal infections. The costs of foodborne diseases to the industry can be significantly high: e.g. US$10–83 billion in USA[11] and >AU$1.2 billion annually in Australia[12].

To reduce antibiotic use in the control of the above-mentioned pathogens in aquaculture farms alternative measures, in particular, those of biological origin, are being sought by the industry. One such measure is the use of bacteriophages that are specific to the disease-causing bacteria (Table 1). Phage therapy so far has displayed encouraging results in aquaculture settings via the use of diverse types of administrations: (1) direct application of phage suspensions in water; (2) oral administration of phages mixing with food; and (3) injections[13,14] (Table 2).

At the University of the Sunshine Coast (USC) in Queensland, Australian research in this field has also been carried out over the past 10 years and specific examples are listed below:

(1) Research study jointly conducted with the USC and the Research Institute for Marine Fisheries, Hai Phong and the Research Institute for Aquaculture No. 2, Ho Chi Minh, Vietnam, Le *et al*.[19] was able to reduce the incidence of disease due to *Aeromonas hydrophila* that causes Motile Aeromonas Septicemia (MAS) in Striped Catfish (*Pangasianodon hypophthalmus*). It is one of the most important farmed fish species in the South East Asia Pacific region including Vietnam, Thailand, Cambodia, Laos and, more recently, the Philippines and Indonesia[21]. In 2015, Vietnam supplied 90% of catfish production with a value of US$1.1–1.7 billion; however, an increase in Motile *Aeromonas* Septicemia cases and the detection of antibiotic resistant species of the pathogen has been threatening the productivity of the industry. Thus, the development of world first bacteriophage treatment against *Aeromonas hydrophila* with successful field trials conducted in Vietnam[19] now offers an alternative disease control strategy for the farmers.

(2) One of the main sources of *Vibrio* infections in aquaculture is the use of microalgae infested by the pathogen as feed in the aquaculture tanks. Bacteriophages were again successfully used to eliminate *Vibrio* infestations on microalgae used as a food source for oyster larvae in oyster hatcheries at the USC in a study jointly conducted with the Port Stephens Fisheries Institute in NSW, Australia. The morphology of one of these phages is illustrated in Figure 1*a*.

(3) Two key vectors for potential *Vibrio* spp. contamination in the hatchery include broodstock and seawater[22]. Bacteriophages were again successfully used to treat *Vibrio* infections in Sydney rock oyster larvae (*Saccostrea glomerata*) and this improved oyster survival rate in the USC in a study again jointly conducted with the Port Stephens Fisheries Institute in NSW, Australia. The morphology of one of these phages is illustrated in Figure 1*b*.

(4) Human pathogenic bacteria can contaminate sea-food because of unhygienic handling practices leading to foodborne diseases. This is a particular problem for oysters which are often eaten raw or only lightly cooked which might not remove human pathogens from the product[23]. Again, at the USC, Le *et al*.[20] successfully isolated five different *E. coli* phages and a *Salmonella* phage and treatments of shucked oysters with these phages resulted in significant decrease in the numbers of

Table 1. Possible advantages and disadvantage of biocontrol measures to aquatic bacterial disease[A].

Advantages	Disadvantages
Abundance in nature, including lytic and lysogenic bacteriophages	Only strong lytic bacteriophages are needed for phage therapy
Treatment does not require repeated administration	Difficult to extrapolate from *in vitro* treatment to *in vivo* expectation
Narrow host range can provide an effective treatment to targeted bacteria, without any effect on other bacteria	Need to identify and isolate the bacterium causing the infection/disease
Rapid process to isolate and select new lytic bacteriophages	Need expertise and experimental setting up and for careful screening to determine the activity spectrum of phages
Administration though feeding, injection and immersion	There might be practical difficulties e.g. injecting large numbers of aquaculture animals
High specificity of killing of pathogens, including antibiotic-resistant bacteria	Phage resistance can be developed by bacteria
Phage resistant colonies might be not pathogenic	Newly isolated phages are required for phage resistant bacteria
No side-effect to microbiota and environment during or after phage application	Phages could transfer virulence factors and other genes coding for undesired traits
Phage cocktails can reduce the phage-resistant-bacteria	All infecting bacteria must be exactly recognised that might have time constrains
Phage therapy might be less expensive than that of antibiotics	More studies in phage therapy might cause additional costs

[A]Adapted and modified from Oliveira *et al*.[13].

Table 2. Examples of successful application of bacteriophage treatments to control fish and shellfish diseases of bacterial origin.

Aquaculture food type	Etiologic agent	Bacteriophage	Source of isolation	Dose and application method used	Results	Reference
Adult oysters	*V. parahaemolyticus*	Phage VPp1	Sewage samples	Oysters were infected with 10^5, 10^6, and 10^7 CFU/mL of *V. parahaemolyticus* in the seawater and treated with three different multiplicity of infection (MOI)[A] values: 0.1, 1 and 10	Depuration at 16°C with 0.1 MOI phage treatment was the best condition for reducing *V. parahaemolyticus* in oysters, which decreased by 2.35–2.76 log CFU/g within 36 h	15
Shrimp larvae (*Litopenaeus vannamei*)	*V. parahaemolyticus*	A3S and Vpms1	A3S and Vpms1 were isolated from shrimp cultures and clams, respectively	MOI values: 0.1, 1, and 10	Phages effectively reduced the mortality rates of larvae caused by *V. parahaemolyticus*, especially when applied at the early larval stage (at 6 h post-infection)	16
Live prey (*Artemia salina*)	*V. alginolyticus*	φSt2 and φGrn1	Water samples from two locations of the north coastline of Crete, Greece	*In vivo* administration of the phage cocktail, φSt2 and φGrn1, at MOI = 100 directly on live prey *A. salina* cultures	93% decrease of presumptive *Vibrio* concentration after 4 h of treatment	17
Shrimp (*Penaeus monodon*)	*V. harveyi*	VHM1 (*Myoviridae*), VHM2 (*Myoviridae*) and VHS1 (*Siphoviridae*)	Water and sediments in Palk Strait, south east coast of India	Post larval stages of shrimp were treated with the test bacterium (10^5 cells/mL) first in laboratory trials followed by single phage treatment (about 10^9 PFU/mL) and phage cocktail treatment (about 10^9 PFU/mL)	Larval survival was 60–88.3% after 96 h in the presence of phage treatment, compared to 26.6–35% larval survival in the control treatments without phage	18
Striped catfish (*Pangasianodon hypophthalmus*)	*Aeromonas hydrophila*	*A. hydrophila* φ-2 and *A. hydrophila* φ-5	River water	All of the fish used in the treatment group were infected intraperitoneally with *A. hydrophila* N17 (3.2×10^6 CFU/fish) and were then immediately injected with a phage cocktail (MOI 0.01, 1 and 100)	The survival rate of catfish with MOI 100 was 100%, compared to the 18.3% survival in the controls unchallenged with the phages	19
Fresh edible Pacific oysters (*Crassostrea gigas*)	*E. coli* strains (JM 109, ATCC 13706 and ATCC BAA 196) and *S. enterica* subsp. *enterica* (ATCC 13311)	Five *E. coli* bacteriophages, φ-Eco1, φ-Eco2, φ-Eco3, φ-Eco5, φ-Eco6 and one for *S. enterica* subsp. *enterica* (ATCC 13311) (φ-S1)	Sewage water at a local sewage treatment plant in the Sunshine Coast region of Queensland, Australia	Bacterial contamination on surface of oyster meat (10^5 CFU/g of oyster meat) was treated with phage cocktail (10^{12} PFU/g oyster meat), along with bacterial control and phage control	A significant reduction for *S. enterica* subsp. *enterica* (ATCC 13311) with the final counts on the agar plate being 1.4 log (CFU/g oyster meat), compared to a control count of 5.7 log (CFU/g oyster meat) was obtained at the end of experiment *E. coli* (ATCC BAA-196) final concentration also indicated a significant difference between treatment of 6.3 log (CFU/g oyster meat) and the control of 7.9 log (CFU/g oyster meat) at 50 h	20

[A]Multiplicity of infection (MOI) is the ratio of infectious agents (e.g. phage or virus) to infection targets (e.g. cell).

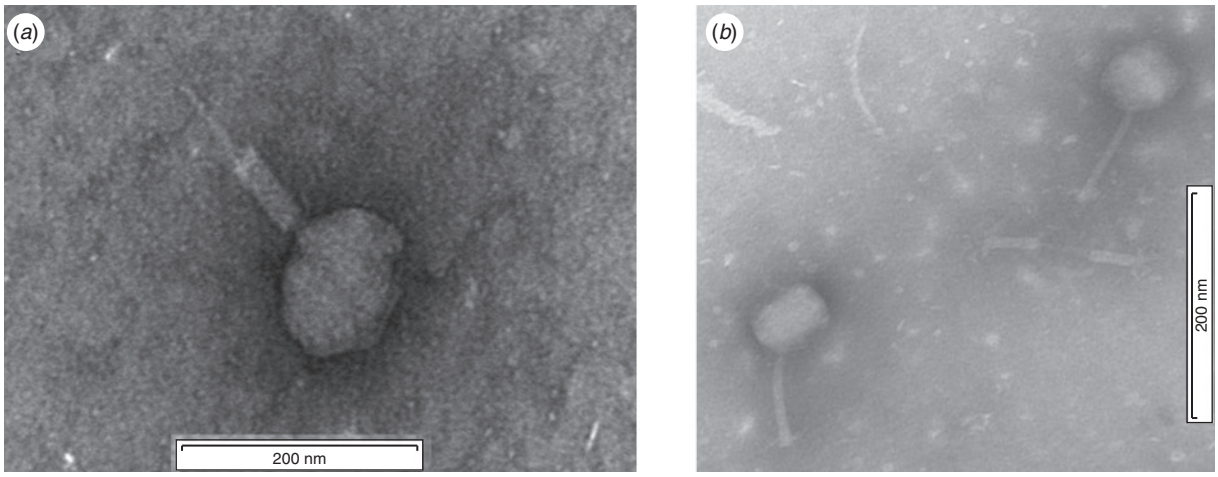

Figure 1. Transmission electron micrograph of *Vibrio alginolyticus* phages isolated in studies 2 (*a*) and 3 (*b*).

E. coli and *Salmonella*. Reduction in the numbers of extended spectrum beta-lactamase resistant *E. coli* strain (ATCC BAA 196) was also achieved[20].

(5) Moreover, off-flavor compound producing bacteria present in the sediments of unlined aquaculture tanks can result in the diffusion of earthy-musty compounds into fish flesh lowering the sale value of the product. Recently, in a joint study between the USC and the SeaFood Team of the Department of Agriculture and Fisheries in QLD, Jonns *et al*.[24] reported a decrease in odours caused by geosmin and 2-methyl-iso-borneol (2-imb) producing streptomycetes when they used streptophages in simulated aquaculture tank experiments in the laboratory. This method provides a safe alternative strategy to farmers whose business is detrimentally impacted by the odour producing bacteria e.g. the barramundi farmers.

Conclusions

The rising incidence of antibiotic resistance in bacteria and problems with antibiotic residues in aquatic environments and aquaculture products, highlight the need for, alternative therapies for control of pathogenic bacteria in aquaculture. Bacteriophage-mediated biocontrol can be one of these alternative methods[15–26]. The cases presented above demonstrate the potential of phage therapy in controlling diseases associated with aquaculture although further data is required for the acceptance and successful application of bacteriophages in aquaculture settings.

There are other factors to be considered before widespread application of bacteriophage therapy can occur such as existence of phage resistant bacteria. Examples include phage-resistant *Streptococcus iniae* causing beta-hemolytic streptococcicosis in Japanese flounder *Paralichthys olivaceus*[27].

Bacteriophages can also mediate toxicity such as the one encountered when *Penaeus monodon* gets infected with *V. harveyi*[28]. Accidental introduction of lysogenic phages was pointed out as an inherent risk for shrimp farmers[29]. *V. harveyi* Siphophage 1 (VHS1) was found to lose its ability to lyse cells but retained its ability to lysogenise after boiling for 10 min. Accordingly, cooking of crustaceans may not be sufficient for full inactivation of phages that might be present in the seafood thus resulting in lysogenic conversions[29].

In-depth understanding on the fascinating interactions between the host and bacteriophages will facilitate development of effective management systems including the use of several techniques in rotation including the bacteriophage therapy[13].

Conflicts of interest

The authors declare no conflicts of interest.

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