ISOLATION AND CHARACTERIZATION OF BACTERIOPHAGE FOR VIBRIOSIS THERAPY IN FISH

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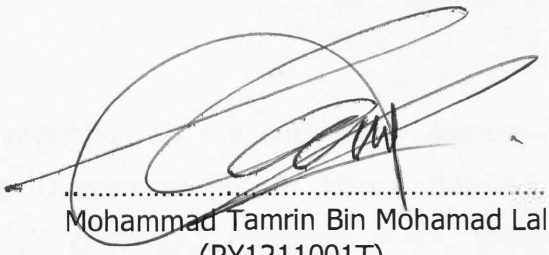

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ABSTRACT

Bacterial infections in aquaculture are commonly treated using antibiotics. However, due to health concern and environmental issues, new control strategies for bacterial diseases are needed. Therefore, this study was conducted to isolate and characterize bacteriophage that are potentially be used as therapy for fish bacterial diseases. Four species of bacterial pathogens (*Vibrio alginolyticus*, *V. harveyi*, *V. parahaemolyticus* and *Photobacterium damselae*) were targeted for bacteriophage isolation. Each bacteriophage isolate was spotted onto different bacterial pathogens (*V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus* and *Ph. damselae*) lawns. The bacteriophage morphology was determined using TEM and the whole genome sequence of bacteriophage was achieved using Illumina sequencing and *de novo* assembly. The stability of the bacteriophage was evaluated on different levels of pH, temperatures and bile concentrations. The bactericidal effect of the bacteriophage was evaluated using the *in vitro* co-culture method. In addition, the toxicity of the bacteriophage was evaluated against brine shrimp (*Artemia* sp.) and Asian seabass (*Lates calcarifer*) juveniles. This study has successfully isolated bacteriophage which were effective against *V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus*. The bacteriophage isolates exhibited high specificity to its host with exception to *V. harveyi* phage that was also capable of infecting *V. parahaemolyticus* ATCC 17802. All phage isolates were classified under the double stranded DNA phage. The TEM analysis revealed that the *V. alginolyticus* phage, *V. harveyi* phage and *V. parahaemolyticus* phage were belong to the Family of *Myoviridae*, *Myoviridae* and *Siphoviridae*, respectively. The complete genome of *V. alginolyticus* phage was estimated at 248,088 bp and has high homology to *Vibrio* phage VH7D. Meanwhile, *V. parahaemolyticus* phage genome was 56,637 bp and hypothetically novel. Interestingly, all the phages possess methylated genome. The bioinformatics analyses revealed that the phage genomes have low significant homologies to vibrio virulent genes and toxin related proteins. All phage isolates were stable at 50 °C but completely deactivated at temperatures higher than 60 °C. The phage also stable at wide range of pH (4-9?) and high bile concentrations. Further analysis showed that the *V. parahaemolyticus* phage required high level of multiplicity of infection (MOI 100) to suppress the growth of its host but V. *harveyi* and *V. alginolyticus* phages required low MOI (0.01) to achieve similar effect. The findings of this study showed that the characteristics of the bacteriophage complied with the phage therapy requirement whereby all phages exhibited bactericidal effect and highly specific. The methylated genome allows the bacteriophage to survive from the defence mechanisms of the host bacteria. Lack of virulence genes prohibits the phage from contributing virulence to host bacteria through horizontal gene transfer. Furthermore, the phages were stable in both acidic and alkaline conditions which make them withstand the extreme condition of the gastrointestinal environment during therapy through oral administration. Most importantly, the bacteriophage were not toxic to the target animals. With these characteristics, the isolated phages seem beneficial for therapeutic use against vibriosis in aquaculture.

ABSTRAK

PEMENCILAN DAN PENGENALPASTIAN BAKTERIOFAJ UNTUK TERAPI VIBRIOSIS IKAN

Jangkitan bakteria di akuakultur pada umumnya dirawat menggunakan antibiotik. Namun, penggunaanya yang boleh menyebabkan masalah kesihatan dan menjejaskan alam sekitar memerlukan strategi kawalan jangkitan bakteria yang baru. Oleh itu, kajian ini dilakukan untuk memencil dan mengenalpasti bakteriofaj yang berpotensi untuk digunakan bagi tujuan terapi. Empat spesies bakteria pathogen (Vibrio alginolyticus, V. harveyi, V. parahaemolyticus dan Photobacterium damselae) digunakan untuk tujuan pemencilan bakteriofaj. Setiap isolat bakteriofaj diuji ke atas hamparan bakteria pathogen. Morfologi bakteriofaj tersebut ditentukan menggunakan TEM dan penjujukan keseluruhan genom bakteriofaj dihasilkan menggunakan penjujukan Illumina dan pemasangan genome de novo. Tahap kestabilan bakteriofaj dikaji pada tahap pH, suhu dan kepekatan hempedu yang berbeza. Kesan bakterisidal bakteriofaj ditentukan menggunakan ujian ko-kultur secara in vitro. Kesan toksik bakteriofaj pula ditentukan menggunakan ujian toksik terhadap anak udang (Artemia sp.) dan ikan siakap (Lates calcarifer). Kajian ini berjaya memmencilkan bakteriofaj yang berkesan melawan V. alginolyticus, V. harveyi dan V. parahaemolyticus. Isolat bakteriofaj tersebut amat spesifik terhadap perumahnya kecuali pada ioslat bakteriofaj V. harveyi yang boleh menjangkiti V. parahaemolyticus ATCC 17802. Semua isolate faj (V. alginolyticus, V. harveyi and V. parahaemolyticus) adalah faj DNA dwibebenang. Analisis TEM menunjukkan faj V. alginolyticus, faj V. harveyi and faj V. parahaemolyticus masing-masing berada pada Famili Myoviridae, Myoviridae and Siphoviridae. Jujukan genom lengkap bagi faj V. alginolyticus dianggarkan pada 248,088 bp dan homolog kepada Vibrio phage VH7D. Manakala, genom V. parahaemolyticus adalah 56,637 bp dan berkemungkinan novel. Menariknya, kebanyakan faj tersebut memiliki genom bermetil. Analisa bioinfomatik menunjukkan genom-genom tersebut memiliki homolg yang rendah terhadap gen virulen dan protein toksin vibrio. Semua faj adalah stabil pada suhu 50 °C tetapi tidak aktif pada suhu lebih tinggi dari 60 °C. Faj tersebut stabil pada julat pH yang besar (4-9) dan boleh bertoleransi pada tahap kepekatan hempedu yang tinggi. Analisis lanjut menunjukkan bahawa Faj V. parahaemolyticus memerlukan MOI yang tinggi (MOI 100) untuk membantutkan pertumbuhan perumahnya, namun, faj V. harveyi dan V. alginolyticus boleh membantutkan pertumbuhan perumahnya pada MOI yang rendah (MOI 0.01). Dapatan kajian ini menunjukkan bakteriofaj tersebut menepati kriteria-kriteia untuk calon terapi dimana ia menunjukkan aktiviti bakterisidal yang tinggi dan amat spesifik. Genom bermetil juga membolehkan bakteriofaj bermandiri dari mekanisma pertahanan perumah. Gen virulen yang tidak dikesan menghadkan peningkatan tahap virulen bakteria melalui perpindahan gen. Selain itu, faj tersebut stabil dalam keadaan berasid dan beralkali membolehkan mereka bertoleransi dengan keadaan ekstrem gastrousus ikan selepas pemberian secara oral. Seterusnya, faj tersebut tidak toksik pada haiwan sasaran. Kesimpulan dari sifat-sifat yang dinyatakan, faj yang dipencilkan dalam kajian ini mungkin berfaedah untuk kegunaan terapeutik menentang vibriosis di akuakultur.

LIST OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

°C	Degree Celcius
µl	microliter
OD_{600}	Optical Density at 600 nm
%	Percent
mM	Milimolar
ml	Mililiter
l	Liter
xg	Times gravity
M	Molar
ng μl^{-1}	Nanoram per microliter
nm	Nanometer
φ	Bacteriophage
Φ	Bacteriophage
ϕ	Bacteriophage
ψ	Bacteriophage
cfu ml^{-1}	Colony forming unit per mililiter
pfu ml^{-1}	Plaque forming unit per mililiter

LIST OF APPENDICES

CHAPTER 1

GENERAL INTRODUCTION

1.1 Common Bacterial Pathogens in Marine Fish Aquaculture

Fish in captivity as well as in the natural habitat are exposed to many kinds of bacterial diseases including as vibriosis, streptococcosis and bacterial kidney disease (BKD) (Toranzo *et al.*, 2005). Vibriosis is one of the bacterial diseases which often occurs in aquaculture. Vibriosis may cause by various *Vibrios* such as *V. anguillarum*, *V. ordalii*, *V. salmonicida*, *V. vulnificus*, *V. harveyi*, *V. alginolyticus*, *V. cholerae*, *V. fischeri*, *V. furnisii*, *V. ichthyoenteri*, *V. logei*, *V. pelagius*, *V. splendidus*, *V. tapetis* or *V. wodanis* (Toranzo *et al.*, 2005; Won and Park, 2008; Austin and Austin, 2007). The outbreak of vibriosis has been reported to occur worldwide involving many marine organisms (Austin and Austin, 2007) and also freshwater fishes (Geng *et al.*, 2014). Fish affected by this disease generally shows typical signs of haemorrhage on the base of fins, exophthalmia, corneal opacity and skin lesions. Meanwhile, the moribund fish will experience severe anemia which manifested by pale gills (Toranzo *et al.*, 2005). Study by Ransangan and Mustafa (2009) showed that *V. harveyi* is responsible for mortality in Asian seabass (*Lates calcarifer*) cultured in Sabah, Malaysia.

Streptococcosis had been reported both in freshwater and marine fish aquaculture. Although it can be caused by many *Streptococcus* species, most of the infections in marine aquaculture are due to *Streptococcus iniae* (Musa *et al.*, 2007). Infected fish normally showed meningoencephalitis, panophthalmitis, skin lesion, necrosis, corneal opacity and hemorrhage (Musa *et al.*, 2007). Streptococcosis can easily be transmitted through contact with infected fish or contaminated feeds (Musa *et al.*, 2007).

Photobacterium damselae is a marine bacterium that causes infection in a variety of marine fish (Rivas *et al.*, 2013). Fish species which are reported to be affected by this pathogen include rainbow trout (Pedersen *et al.*, 2009), seabass

(Labella *et al.*, 2006) and turbot (Fouz *et al.*, 1992). This pathogen is reported to causing wound infections and haemorrhagic septicemia in fish. (Rivas *et al.*, 2013).

1.2 Treatments Option for Bacterial Diseases

Fish diseases caused by bacteria are commonly treated with antibiotics. However, due to health concern and environmental issues, the use of antibiotics is no longer accepted in many countries including Malaysia (Musa *et al.*, 2008). Studies also showed that rampant use of antibiotic can promote the development of antibiotic resistant bacteria in net cage aquaculture environment (Tendencia and de la Pena, 2001). Hence, there is a need for development of noble strategies which are harmless to both consumers and environment, in fighting for bacterial pathogens in aquaculture.

The use of vaccines in aquaculture has been shown to successfully protect fish against bacterial diseases, such as vibriosis (Sun *et al.*, 2009), edwardsiellosis (Liu *et al.*, 2005), furunculosis (Gudmundsdóttir and Björnsdóttir, 2007), streptococcosis (Heath and Feldman, 2005) and pasteurellosis (Andreoni and Magnani, 2014). According to Collado *et al.* (2000), vaccine application was better solution against vibriosis. However, vaccine is only promoting the fish immune system to resist the bacterial infection without controlling the proliferation of the target bacteria itself. Therefore, other strategy to control the target bacteria is necessary.

The increasing interest in the application of bacteriophages in aquaculture is something worthy to investigate (Nakai and Park, 2002). Due to its host specificity, bacteriophages normally do not disturb the natural bacterial flora inside the fish. Therefore, isolation of bacteriophages which have the ability to kill fish bacterial pathogens may provide new avenue for diseases control in aquaculture.

1.3 Bacteriophage

Bacteriophage are viruses which prey on bacteria (Gillis and Mahillon, 2014). Similar to other viruses, they are absolute parasitic to bacteria (Kutter and Sulakvelidze, 2005). Bacteriophage were first discovered by Federick Twort and

Felix d'Herelle in 1915 and 1917, respectively (Duckworth, 1976). Independently, Felix d'Herelle characterized this virus and named as bacteriophage, meaning "bacterial eater" (D'Herelle, 1917). The subsequent decades, researchers continue to examine the nature of the bacteriophage. In fact, the bacteriophage have been used as model microorganism to investigate the various aspect of viruses (Keen, 2015), such as virion structure, genetics and viral replication system. Hershey and Chase (1952) reported that the DNA was the carrier of genetic information in bacteriophage. The T4 bacteriophage was also used as a tool to study the discontinuous replication of DNA by Okazaki *et al.* (1968). The bacteriophage lambda has been extensively used for a range of studies including understanding of gene regulation (Ptashne *et al.*, 2004) and vector for gene analysis (Chauthaiwale, 1992). In addition, the extensive study on bacteriophage genome has provide the insight into the identification of novel biochemical mechanisms (Miller *et al.*, 2003a).

The intensive study on therapeutic use of bacteriophage began in 1920 (Carlton, 1999). After the discovery of the first antibiotic, Penicillin in 1928 (Garrod, 1947), the study of therapeutic possibilities of bacteriophage was abandoned in favour of the wider usage of antibiotics (Gill and Hyman, 2010). However, the research on bacteriophage continued in the Eastern Europe and former Soviet Union (Sulakvelidze *et al.*, 2001). The lack of international peer review and limited number of English articles have somehow contributed to unavailability of the progress of these works to the international scientific communities. The interest in bacteriophage therapy was only revived in recent years following the rampant occurrence of antibiotic resistant bacteria (Keary *et al.*, 2013).

1.3.1 Taxonomy of Bacteriophages

The initial classification of bacteriophage was based on the different in host specificities (Nelson, 2004). With the advent of electron microscope, the bacteriophage was classified using morphology. To date, approximately 96% of the bacteriophage belong to the order Caudovirales have been successfully examined via electron microscopy (Ackermann, 2003). The current report on the taxonomy of bacteriophage is listed in Table 1.1.