Virulent bacteriophage of *Edwardsiella ictaluri* isolated from kidney and liver of striped catfish *Pangasianodon hypophthalmus* in Vietnam

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ABSTRACT: Striped catfish *Pangasianodon hypophthalmus* farmed in the Mekong Delta, Vietnam, represents an important contribution to Vietnamese aquaculture exports. However, these fish are affected by frequent disease outbreaks across the entire region. One of the most common infections involves white spots in the internal organs, caused by the bacterium *Edwardsiella ictaluri*. In this study, a virulent phage specific to *E. ictaluri*, designated MK7, was isolated from striped catfish kidney and liver samples and characterized. Morphological analysis indicates probable placement in the family *Myoviridae* with a 65 nm icosahedral head and a 147×19 nm tail. A double-stranded DNA genome of approximately 34 kb was predicted by restriction fragment analysis following digestion with *Sma*I. The adsorption affinity (k_a) of the MK7 phage was estimated as 1.6×10^{-8} ml CFU^{-1} min^{-1}, and according to a 1-step growth curve, its latent period and burst size were ~45 min and ~55 phage particles per infected host cell, respectively. Of the 17 bacterial strains tested, MK7 only infected *E. ictaluri*, although other species of *Edwardsiella* were not tested. *E. ictaluri* was also challenged *in vitro*, in both broth and water from a striped catfish pond and was inactivated by MK7 for 15 h in broth and 51 h in pond water. Thus, initial characterization of phage MK7 indicates its potential utility as a biotherapeutic agent against *E. ictaluri* infection in striped catfish. This is the first report of a lytic phage specific to an important striped catfish pathogen.

KEY WORDS: Phage therapy · Striped catfish · Characterization · *Edwardsiella ictaluri* · Mekong Delta · Antibiotic resistance

1. INTRODUCTION

Striped catfish or Vietnamese catfish *Pangasianodon hypophthalmus* is a native freshwater catfish of the Mekong Delta, Vietnam (MKDVN). This catfish is an important component of national aquaculture exports, and accounts for 90% of the 1.4 million t of striped catfish produced globally (De Silva et al. 2011). In addition, frozen striped catfish fillet is among the predominant fish products exported from Vietnam. According to Vietnam's Office of Statistics, in 2017, aquaculture exports generated US$8.3 billion dollars for the nation's economy, with striped catfish (accounting for 22% of such exports) being the second most exported item (GSO Vietnam 2017).

One of the major factors affecting the sustainability of the MKDVN aquaculture industry is the annual cycle of pathogenic bacterial infections, the most common of which is white spots in the internal organs caused by *Edwardsiella ictaluri*. This disease first appeared among striped catfish in Vietnam in 1998 and is associated with a high mortality rate (up to 90%) (Crumlish et al. 2002, Dang & Nguyen 2010). The use of antibiotics as the major preventative and

curative treatment for this disease has since become common in the region. However, such control efforts have recently proven inadequate due to the development of antibiotic resistance by *E. ictaluri*. Quach et al. (2014) showed that the antibiotic resistance rate of *E. ictaluri* isolates from diseased striped catfish was 100% for trimethoprim/sulfamethoxazole, more than 80% for enrofloxacin, chloramphenicol, streptomycin, and florfenicol, and approximately 50% for cefalexin, norfloxacin, neomycin, gentamycin, and cefotaxime. This has raised concerns regarding the long-term efficacy of antibiotic treatment in the commercial production of striped catfish. In addition, residual antibiotics at levels above approved limits in frozen striped catfish fillets may affect consumers' health. Due to these adverse impacts, there is an urgent need to identify more effective solutions to replace antibiotics.

Phage therapy, i.e. the use of lytic phages as a treatment for pathogenic bacterial infections, has existed since the 1930s. However, this method has only attracted serious attention in the aquaculture industry within the last 30 yr, driven by the widespread increase in antibiotic resistance. The efficacy of phage therapy for bacterial diseases in fish and shellfish has been established, e.g. in the treatment of *E. tarda* causing edwardsiellosis in loach *Misgurnus anguillicaudatus* (Wu & Chao 1982), *Lactococcus* spp. causing lactococcosis in yellowtail *Seriola quinqueradiata* (Nakai et al. 1999), *Aeromonas salmonicida* causing furunculosis in brook trout *Salvelinas fontinalis* (Imbeault et al. 2006), and *A. hydrophila* causing tail and fin rot in loach (Wu et al. 1981, Jun et al. 2013). However, there have been no reports of the isolation and characterization of a virulent bacteriophage specific to *E. ictaluri* as the causative agent of white spots in the internal organs of striped catfish. The present study represents the first report of the isolation and characterization of an *E. ictaluri*-specific virulent phage from striped catfish in the MKDVN. The efficacy of this phage in inactivating *E. ictaluri* in tryptic soy broth (TSB) and striped catfish pond water was also examined.

2. MATERIALS AND METHODS

2.1. Phage isolation

Striped catfish kidney and liver samples were obtained from striped catfish farms (Tien Giang province, MKDVN) and transferred to the laboratory under cold conditions for phage isolation. The samples were homogenized and added to a log-phase *Edwardsiella ictaluri* CT1 culture (Crothers-Stomps et al. 2010). The mixture was then incubated overnight at 30°C with rotation at 150 rpm. An aliquot was subsequently taken and centrifuged at $9727 \times g$ at 4°C for 5 min. The resulting supernatant was passed through a 0.22 µm filter, and the filtrate subjected to a plaque assay. Briefly, a mixture of 100 µl of filtrate and 200 µl of log-phase *E. ictaluri* CT1 culture was added to 3 ml of molten 0.5% brain heart infusion (BHI) agar (maintained at 37°C) and poured over a 1.5% Luria–Bertani (LB) agar plate. After incubation overnight at 30°C, a single transparent plaque was picked from the plate, suspended in SM buffer (100 mM NaCl, 10 mM $MgSO_4$, 0.01% gelatin, 50 mM Tris-HCl; pH 7.5), incubated overnight at 4°C, and passed through a 0.22 µm filter. The resulting filtrate was subjected to the above protocol 3 times in succession to purify the phage. The purified phage stock was prepared as follows. After the plaque assay, the molten agar lawn was mixed with 2 ml of SM buffer and 200 µl of chloroform. This mixture was incubated at room temperature (~30°C) for 2 h, before being centrifuged at $9727 \times g$ for 5 min. The resulting supernatant was passed through a 0.22 µm filter to obtain the phage stock, the concentration of which was established using the plaque assay described above.

2.2. Restriction analysis of phage nucleic acids

Phage genomic DNA was extracted using the Phage DNA Isolation Kit (Norgen Biotek). It was then digested using restriction enzyme *Sma*I (Thermo-Fisher Scientific) at 30°C for 16 h. The DNA fragments were subjected to electrophoresis on 1% agarose gel at 120 V for 70 min. Thus, the genome size was estimated (Walakira et al. 2008, Higuera et al. 2013).

2.3. Transmission electron microscope examination

A highly concentrated suspension of phage (10^{10} plaque-forming units [PFU] ml^{-1}) was prepared as previously described (Ackermann 2009a). The phage sample was then negatively stained with 5% uranyl acetate and observed by transmission electron microscope (JEOL JEM-1010) operating at a voltage of 79 kV and an instrumental magnification of 50 000 at the Vietnam National Institute of Hygiene and Epidemiology.

2.4. Phage adsorption assay

E. ictaluri CT1 was incubated at 30°C in TSB until the culture reached an optical density at 600 nm (OD_{600}) of 0.1 (ca. 10^7 colony-forming units [CFU] ml^{-1}). MK7 phage was added to the culture at a multiplicity of infection (MOI) of 0.01 (phage:host), and the mixture incubated at 30°C. Aliquots were removed at 5 min intervals and were immediately centrifuged at $14\,000 \times g$ for 5 min at 4°C to obtain filtrate, the phage titer of which was determined using the plaque assay described above. The proportion of unbound phages was determined by comparing the concentration of free phages in the filtrate to the initial phage concentration. Phage titer at 0 min was defined as being 100%. The experiment was conducted in triplicate.

2.5. One-step growth curve

The 1-step growth curve of phage MK7 was established according to the method of Pajunen et al. (2000), with some modifications. An *E. ictaluri* CT1 culture in TSB was incubated at 30°C with rotation at 120 rpm until its OD_{600} reached 0.1 (ca. 10^7 CFU ml^{-1}). The phage was then added at an MOI of 0.01. This mixture was incubated as above for 10 min and subsequently centrifuged at $2432 \times g$ for 5 min at 4°C. The resulting pellet was resuspended in the same volume of TSB and incubated as above. Samples were taken every 5 min and diluted 100-fold in SM buffer on ice. The diluted samples were centrifuged at $14\,000 \times g$ for 5 min at 4°C, and the supernatant was used for phage titration. The latent period and burst size of the phage were determined according to a previously described method (Pajunen et al. 2000). The experiment was conducted in triplicate.

2.6. Host range determination

The host range of phage MK7 was determined using various strains of bacteria (Table 1), the susceptibility of which was examined using a modified drop plaque assay (Namura et al. 2008). A 100 μl aliquot of an overnight bacterial culture was added to 3.0 ml of molten 0.5% BHI agar (maintained at 37°C), and this mixture poured over a 1.5% LB agar plate. After 2 min, 2 μl of MK7 stock (ca. 10^9 PFU ml^{-1}) was dropped onto each plate. The plates were incubated overnight at 30°C and subsequently observed for clear zones on the bacterial lawn.

Table 1. List of bacterial strains and host range of MK7. (+) susceptible; (−) not susceptible; CTU: College of Aquaculture and Fisheries, Can Tho University; RIA2: Research Institute for Aquaculture No. 2; ATCC: American Type Culture Collection; MTCC: Microbial Type Culture Collection

Bacterial strain	Reference/ source	Plaque formation
Edwardsiella ictaluri CT1	CTU	+
Edwardsiella ictaluri E2	CTU	+
Edwardsiella ictaluri E3	CTU	+
Aeromonas hydrophila A1	CTU	−
Aeromonas hydrophila VTS1	RIA2	−
Aeromonas dhakensis VTS11	RIA2	−
Aeromonas veronii VTS12	RIA2	−
Aeromonas sorbia VTS08	RIA2	−
Vibrio parahaemolyticus VTS	RIA2	−
Escherichia coli K12	ATCC	−
Escherichia coli O157:H7	ATCC	−
Escherichia coli C	ATCC	−
Salmonella typhimurium 14028	ATCC	−
Pseudomonas aeruginosa BK	MTCC	−
Bacillus cereus 11778	ATCC	−
Staphylococcus aureus 25923	ATCC	−
Listeria monocytogenes 19111	ATCC	−

2.7. Challenge test in broth

The host bacterial culture in TSB was incubated at 30°C with rotation at 40 rpm until its OD_{600} reached 0.1 (ca. 10^7 CFU ml^{-1}). The culture was then divided into 2 aliquots, of which, one was mixed with phage MK7 lysate at an MOI of 2.0, and the other was not. The mixtures were incubated as described above, with samples being taken every hour for OD_{600} measurements. Another control was prepared in a similar manner by adding phage MK7 to TSB. The samples were passed through 0.22 μm filters to obtain filtrate, the phage titer of which was determined using a plaque assay as described above. The experiment was conducted in triplicate.

2.8. Challenge test in pond water

The inactivation of host bacterial cells by phage MK7 was also tested in a striped catfish pond water sample. The host bacterial culture in TSB was incubated at 30°C with rotation at 40 rpm until its OD_{600} reached 0.1 (ca. 10^7 CFU ml^{-1}). The culture was then centrifuged at $608 \times g$ for 5 min at 4°C and the supernatant discarded. The resulting pellet was resuspended in the same volume of sterilized pond water. Centrifugation and resuspension were repeated to eliminate any residual TSB, with the final pellet being

resuspended and serially diluted in sterilized pond water to attain a bacterial concentration of approximately 10^5 CFU ml^{-1}. The bacterial suspension was divided into 2 aliquots, of which, one was mixed with phage MK7 lysate at an MOI of 2.0, and the other was not. The mixtures were incubated as described above. Samples were taken at 1 or 2 h intervals, serially diluted, and spread on tryptic soy agar to measure cell concentration. Another control was prepared in a similar manner by adding the phage to sterilized pond water. The samples were passed through 0.22 µm filters to obtain filtrate, the phage titer of which was determined using a plaque assay as described above. The experiment was conducted in triplicate.

3. RESULTS

3.1. Lytic activity of phages

Several phages were isolated and purified, of which phage MK7 resulted in clear and large plaques (ca. 2–3 mm in diameter). The other phages were not chosen since they resulted in small plaques (ca. 0.5 mm in diameter). The phage MK7 stock prepared had a concentration of approximately 10^{10} PFU ml^{-1}. Phage adsorption activity with respect to host cells is shown in Fig. 1. Ten minutes after adding the phage to the bacterial suspension, the proportion of unbound phages was measured as approximately 20%, indicating that 80% of phage particles had infected the host cells. The phage adsorption affinity (k_a) was also determined. In this study, phage MK7 was added to the host cell culture at an MOI of 0.01, and the initial host cell concentration was approximately 10^7 CFU ml^{-1}. Ten minutes after their addition to the bacterial suspension, 80% of phage MK7 particles had adsorbed to host cells. Following the method described in a previous study (Yoichi et al. 2005), the k_a was thus estimated as 1.6×10^{-8} ml CFU^{-1} min^{-1}. To determine the latent period and burst size of the phage, a 1-step growth experiment was conducted. The latent period was found to be approximately 45 min and the burst size around 55 phage particles per infected host cell (Fig. 2).

3.2. Genome size and morphology of phage MK7

The phage genome is double-stranded DNA, as it was digested by the restriction endonuclease *Sma*I (Fig. 3A). The genome size is approximately 34 kb. Based on the morphological analysis by transmission electron microscopy (Fig. 3B), MK7 phage was placed in the family *Myoviridae* (Ackermann 2009b). The phage has an icosahedral head that is 65 nm in diameter. Its tail is 147 nm in length and 19 nm in width.

3.3. Host range of phage MK7

The host range of phage MK7 was determined using 17 bacterial strains (Table 1). This phage formed clear zones in cultures of 3 strains: *Edwardsiella ictaluri* CT1, *E. ictaluri* E2, and *E. ictaluri* E3. These strains were previously isolated from samples of diseased striped catfish from the MKDVN. However, none of the other bacteria tested were susceptible to phage MK7. This might be indicative of host specificity of MK7 to *Edwardsiella* although no additional *Edwardsiella* species were tested in this study.
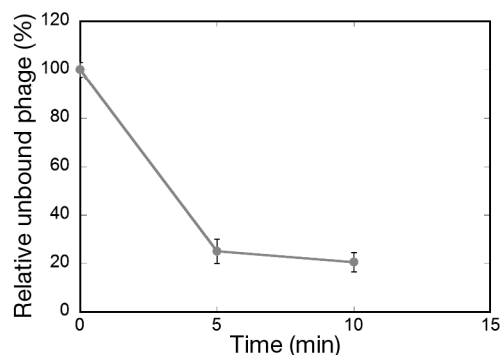

Fig. 1. Phage adsorption on *Edwardsiella ictaluri* cells. Relative phage titers in the supernatant of the host bacterium-phage mixture were measured. Phage titer at 0 min was ca. 10^5 PFU ml^{-1} and was defined as being 100%. Error bars indicate 95% confidence intervals for the averaged values (n = 3)
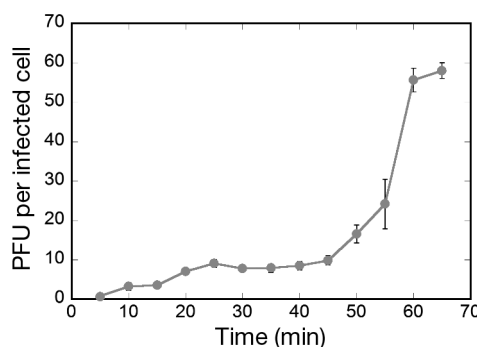

Fig. 2. One-step growth curve of phage MK7 in *Edwardsiella ictaluri* cells at 30°C. Error bars indicating 95% confidence intervals for the averaged values (n = 3) are not graphically detectable as the intervals are too narrow
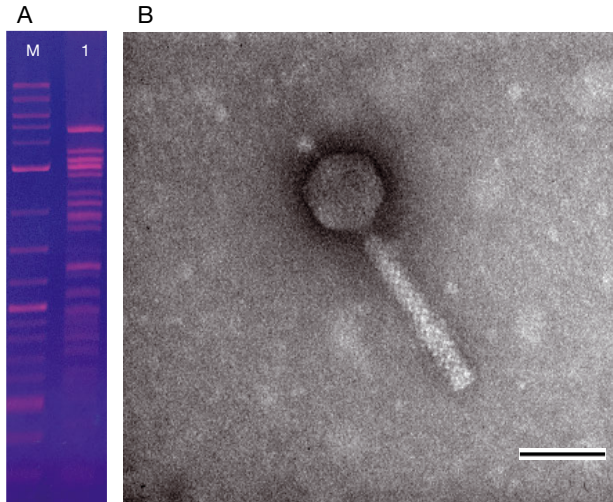
Fig. 3. (A) M: marker Quick-Load® 2-Log DNA Ladder, NEB. Lane 1: restriction pattern of the phage genome with *Sma*I resolved by agarose gel electrophoresis. (B) Electron micrograph of phage MK7. Scale bar = 100 nm

3.4. Inactivation of *E. ictaluri* in broth by phage MK7

Phage MK7 was further evaluated for its capacity to restrict the growth of *E. ictaluri* in TSB. As shown in Fig. 4, during the first hour of incubation, an increased OD_{600} was observed in both treatment groups (with or without the phage). However, the OD_{600} of the suspension of host cells and phages increased to a lesser extent than that of the negative control, which contained only host cells. This implies that a proportion of the host cells had already been infected and lysed by phage MK7 by this point. However, the OD_{600} of the bacterium–phage solution decreased sharply after incubation for 2 h, while that of the negative control continued to increase. The bacterium–phage suspension was established at an MOI of 2.0. The infection of most of the host cells and their lysis by phages thus resulted in the sharp decrease of the suspension's OD_{600}. The transparency of the bacterium–phage solution for approximately 15 h indicated the efficacy of phage MK7 in inactivating the host cells, while the turbidity of the negative control continued to increase over the course of the experiment. After around 15 h, the OD_{600} of the bacterium–phage solution increased, reflecting the growth of phage-resistant bacteria.

Together with the lysis of host cells, new phage particles were generated. Fig. 5 shows the time course of phage titer during the experiment. Phage concentration increased sharply during the first 5 h in which phage and host cells were incubated together. It subsequently increased slightly from 5 to 7 h of incuba-
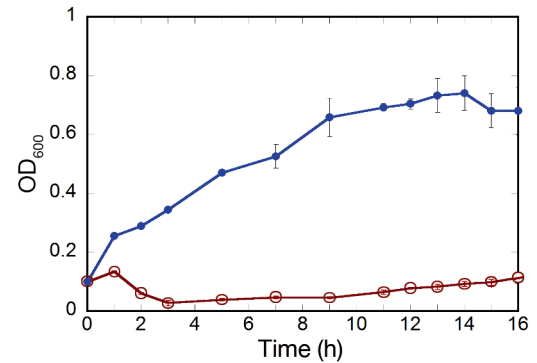

Fig. 4. Changes in optical density at 600 nm (OD_{600}) during inactivation of *Edwardsiella ictaluri* CT1 by phage MK7 in TSB at 30°C (multiplicity of infection [MOI] of 2.0) (open circles). The negative control lacked phages (closed circles). Error bars indicating 95% confidence intervals for the averaged values (n = 3) are not graphically detectable as the intervals are too narrow
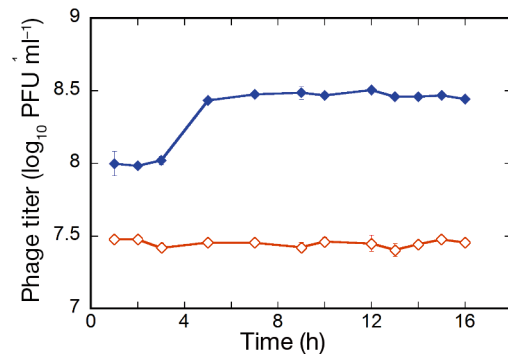

Fig. 5. Time course of phage MK7 titer during the challenge test in tryptic soy broth (TSB). The number of phages in the host bacterium–phage mixture increased (closed diamonds), whereas that in the negative control remained largely constant (open diamonds). Error bars indicating 95% confidence intervals for the averaged values (n = 3) are not graphically detectable as the intervals are too narrow

tion, before stabilizing. At this stage, most of the host cells had already been lysed as a result of the stable number of phages. In the negative control (phages but no host cells), the number of phages remained almost constant during the entire experiment.

3.5. Inactivation of *E. ictaluri* in pond water by phage MK7

A suspension of host cells in sterilized pond water was prepared at an initial concentration of 10^5 CFU ml^{-1}. This concentration is similar to those recorded in ponds containing diseased striped cat-

fish. As shown in Fig. 6A, following challenge with phage MK7, the host bacterial count only increased for the first hour, and sharply decreased over the next 15 h of incubation. The count of viable bacteria was low after 15 h, and remained at this level up to 51 h of challenge. In contrast, the viable bacterial count in the negative control (host cells without phages) remained mostly stable during the experiment. Thus, phage MK7 inactivated *E. ictaluri* in pond water in a highly efficient manner. This inactivation lasted much longer in pond water than in TSB (Fig. 4).

As above, both lysis of host cells and the generation of new phage particles occurred in pond water. Fig. 6B shows the changes in phage titer over the experiment. Phage concentration increased in the mixture of phages and host cells, but remained constant in the negative control (which lacked host cells).
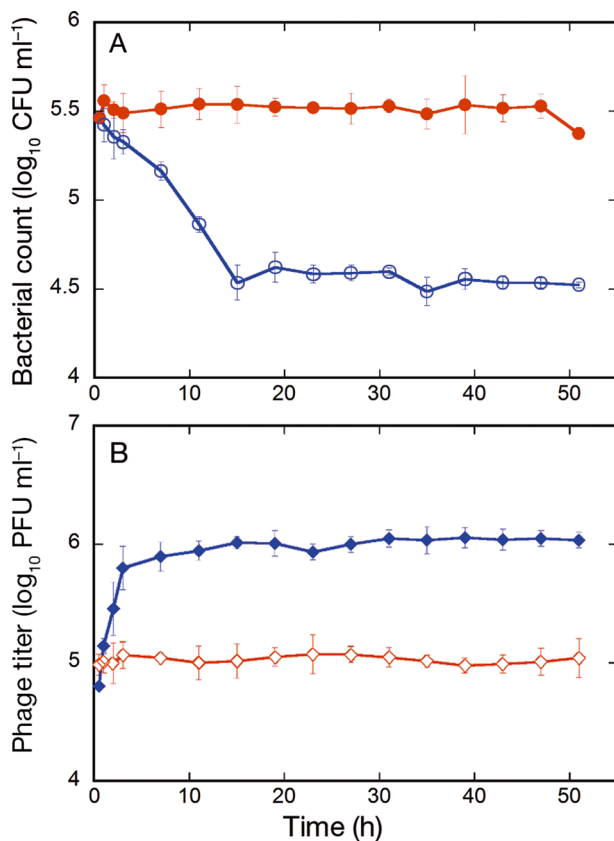

Fig. 6. Time course of host cell count and phage titer during the challenge test in striped catfish pond water at 30°C. (A) Viable *Edwardsiella ictaluri* cell count in the presence (open circles) and absence (negative control; closed circles) of phage MK7. (B) Phage number in the presence (closed diamonds) and absence (negative control; open diamonds) of host bacterial cells. Error bars indicate 95% confidence intervals for the averaged values (n = 3)

4. DISCUSSION

Edwardsiella ictaluri is one of the main causative agents of mass mortality among striped catfish. However, no effective method has been established to control *E. ictaluri* infections, except for the use of antibiotics. The high rates of resistance to antibiotics exhibited by this pathogen have led to a significant reduction in production. Moreover, increased levels of residual antibiotics have been detected in exported stock. Recently, many consignments from leading Vietnamese producers have been rejected by importers in markets such as the USA, Japan, South Korea, Canada, and Russia due to the presence of residual antibiotics in stock at levels higher than the approved limit. Notably, since August 2, 2017, all consignments of imported striped catfish have been tested for residuals of 89 types of antibiotics by the US Food and Drug Administration (Nhân Dân Online 2017).

Phages are considered an alternative to antibiotics for the control of pathogenic bacteria, and phage therapy has been shown to be an effective treatment for bacterial diseases of many types of fish and shellfish (Defoirdt et al. 2011, Richards 2014). However, to the best of our knowledge, no information concerning phage therapy for the treatment of white spots in the internal organs of striped catfish has yet been reported. Walakira et al. (2008) were the first to isolate and characterize a phage specific to *E. ictaluri*, the causative agent of enteric septicemia of channel catfish *Ictalurus punctatus* Rafinesque in the USA. Moreover, Rogge et al. (2013) compared *E. ictaluri* strains isolated from striped catfish in Vietnam with those isolated from US channel catfish, finding the 2 groups to be very different, especially in cell surface lipopolysaccharide structure. This may result in differences in the capacity of phages to infect *E. ictaluri* strains.

In the current study, to investigate the use of phage therapy for the control of *E. ictaluri*, suitable phages were isolated. Many phages corresponding to different bacterial fish pathogens can be isolated from aquaculture water samples (Vinod et al. 2006, Richards 2014, Hoang et al. in press). In the present work, the isolation of a phage specific to *E. ictaluri* was first attempted using water from striped catfish ponds. However, this approach failed, despite having tested 500 2 l samples, implying that the concentration of *E. ictaluri*-specific phages in such pond water is low. This is consistent with the report of Walakira et al. (2008), who resorted to the use of ultrafiltration membranes to concentrate the phages present in the sam-

ples collected. As *E. ictaluri* causes a disease in the internal organs of striped catfish, it seems probable that phages of this bacterium may also be frequently found at these sites. In this study, such a phage (MK7) was successfully isolated from striped catfish kidney and liver samples.

To evaluate the potential of phage MK7 as an antimicrobial agent for the treatment of *E. ictaluri* infection, its lytic activity needed to be clarified. Lytic activity is determined based on 3 main criteria, namely, the adsorption rate, latent period, and burst size. A highly lytic phage is characterized by a short latent period, a high adsorption rate, and/or a high burst size. The adsorption rate of MK7 was approximately 80%, and this phage had an estimated k_a of 1.6×10^{-8} ml CFU^{-1} min^{-1}. This adsorption rate is similar to that of other fish pathogen phages (Yamaki et al. 2014) and coliphages (Tanji et al. 2004, Yoichi et al. 2005). The 1-step growth curve for MK7 revealed a latent period of approximately 45 min and a burst size of around 55 phage particles per infected host cell. This latent period is similar to that of the phages described by Walakira et al. (2008), although the burst size is lower.

The inactivation of host cells is one of the most important factors to consider when evaluating phages as alternative antimicrobial agents. Phage MK7 demonstrated a high capacity to restrict the growth of *E. ictaluri*, maintaining an OD_{600} below 0.1 and greatly increasing in concentration over 15 h in the challenge experiment in TSB. The period of bacterial inactivation by MK7 was longer than that reported for coliphages (6 h according to Morita et al. 2002), *Aeromonas* phage (6 h according to Hoang et al. in press), *Morganella* phage (5 h according to Yamaki et al. 2014), and other fish pathogen phages (Richards 2014). However, the OD_{600} of the bacterium-phage solution did increase after approximately 15 h, indicating the growth of phage-resistant bacteria, the regular emergence of which is the major challenge for phage therapy (Labrie et al. 2010, Oliveira et al. 2012, Mateus et al. 2014). Such resistant bacteria arise via several mechanisms, including alteration of receptors to which phages attach and the development of adaptive immunity through interfering CRISPR sequences (Hyman & Abedon 2010, Labrie et al. 2010, Örmälä & Jalasvuori 2013). There are 2 main approaches to addressing this problem. The first consists of isolating newly mutated phages capable of infecting the phage-resistant bacteria. Co-evolution of phages following the development of resistance by bacteria has been documented previously (Mizoguchi et al. 2003), and this strategy takes considerably less time than that needed to identify a

new antibiotic. The second comprises the application of a phage cocktail to inactivate phage-resistant bacteria (Defoirdt et al. 2011, Richards, 2014).

There have been no publications concerning phage therapy of bacterial diseases of striped catfish. Walakira et al. (2008) described a phage capable of *in vitro* inactivation of *E. ictaluri* isolated from US channel catfish, but the cell surface structure of this bacterium differs from that of *E. ictaluri* isolated from Vietnamese striped catfish (Rogge et al. 2013). There are 2 published genomes of phages of *E. ictaluri* (Carrias et al. 2011, Yasuike et al. 2014). However, those strains infected other types of catfish from striped catfish. They were also isolated from the environmental locations distant from Vietnam. We have compared whole genomic sequences of such phages and found that the similarity of nucleotide sequences were extremely low (data not shown).

In the current study, the inactivation of *E. ictaluri* by phage MK7 in striped catfish pond water was striking, with the viable bacterial cell count remaining low for 51 h. Previous studies have shown that the earliest stage of *E. ictaluri* infection in catfish is an infection of the olfactory sac and brain (Miyazaki & Plumb 1985, Shotts et al. 1986). Therefore, investigation of inactivation of *E. ictaluri* in pond water by phage in the current study is necessary for further prophylaxis and treatment of the disease on farms. Moreover, temperature and pH ranges in pond water suitable for striped catfish range between 25° and 32°C (Duong 2004) and 5.5–9.0 (Le et al. 2017), respectively. Consequently, the stability of the phage under these variable conditions will be evaluated in future studies.

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