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Phage Cocktails to Inactivate *Edwardsiella ictaluri*, an Infectious Agent in Striped Catfish *Pangasianodon hypophthalmus*

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Striped catfish Pangasianodon hypophthalmus farmed in the Mekong Delta, Vietnam, is an important component of national aquaculture exports. However, bacterial diseases result in the decrease of striped catfish production efficiency and the most popular disease is white spots in the internal organs caused by Edwardsiella ictaluri. In this study, four phages specific to E. ictaluri, designated G1, G7, G8 and G9.2, were isolated from striped catfish liver samples and characterized. Morphological analysis indicates probable placement in the Corticoviridae or Tectiviridae family with a 46 – 48 nm icosahedral head and no tails. According to one-step growth curves, their latent period and burst size were 55 – 70 min and 28 – 160 PFU/cell. E. ictaluri was challenged in vitro in broth and was inactivated by single phage for 18 – 20 h. However, a phage cocktail of G7 + G8 was markedly effective in inactivation of the host cells for 45 h. The current study is the first report about success of phage cocktail to inactivate *E. ictaluri*, an infectious agent in striped catfish *Pangasianodon hypophthalmus*.

1. Introduction

Striped catfish or Vietnamese catfish (*Pangasianodon hypophthalmus*) is a native freshwater catfish of the Mekong Delta, Vietnam, which is an important component of national aquaculture exports. According to Vietnam's Office of Statistics, in 2020, aquaculture exports generated 8.6 x 10^9 USD for the nation's economy, in which striped catfish contributed 8.6 x 10^9 USD (VASEP, 2020).

However, bacterial diseases result in the decrease of striped catfish production efficiency and the most popular disease is white spots in the internal organs caused by *Edwardsiella ictaluri*. The disease associates with a high mortality rate, up to 90 % (Dung et al., 2002). Antibiotics have been commonly used to control bacterial infections in human and in aquaculture. However, there have been many disadvantages regarding to the usage of antibiotics such as high prevalence of antibiotic-resistant bacterial strains in hospitals (Mai et al., 2020), in aquaponic systems (Dong et al., 2020), in striped catfish farms (Quach et al., 2014). Quach et al. (2014) reported that antibiotic resistance ratio of *A. hydrophila* isolates in ill striped catfish in the MKDVN was 100 % counted for ampicillin, amoxicillin, cefalexin, trimethoprim/sunfamethoxazol and was 93 % counted for tetracyclin.

For many decades, phages or bacterial viruses have been studied as alternative biological agents to control pathogenic bacterial infections. Phages have been used in USA and some other countries for prevention and treatment of bacterial diseases in plants. Some commercial phage-containing products are available on markets and allowable for usage in organic farming such as Agriphage (OmniLytics, Inc.). In the aquaculture industry, the method become more important in the last forty years with the widespread appearance of drug resistant bacteria, firstly in Loach (Wu et al., 1981) and in other kinds of fishes (Kowalska et al., 2020). The efficacy of phage therapy for bacterial diseases in fish (Doss et al., 2017) and shellfish has been demonstrated (Culot et al., 2020). Usage of phage therapy via different routes for prevention of haemorrhagic septicaemia in striped catfish has been reported such as via oral administration (Dang et al., 2021) or injection route (Le et al., 2018). However, there are not many studies about phages specific to *E. ictaluri* as the causative agent of white spots

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in the internal organs of striped catfish. Hoang et al. (2018) presented the first report of the isolation and characterization of an *E. ictaluri*-specific phage from striped catfish in the MKDVN. *E. ictaluri* was only inactivated by the single phage for 15 h in broth due to the phage-resistant development of the host bacteria. However, there is no study for investigating efficacy of phage cocktails to inactivate striped catfish *E. ictaluri*. In the current study, four phages specific to *E. ictaluri* were isolated and their characteristics such as morphology, latent period, burst size, inactivation activity were investigated. In addition, a mixture of phages (phage cocktail) was demonstrated to be much more effective than single phage in control of *E. ictaluri.*

2. Materials and methods

2.1 Phage isolation

Striped catfish liver samples were obtained from local markets in Ho Chi Minh City and transferred to the laboratory under cold condition for phage isolation. Samples were homogenized and added to a log-phase *E. ictaluri* CT1 culture. The mixture was then incubated overnight at 30 °C with rotation at 150 rpm. An aliquot was subsequently taken and centrifuged at 9,727 ×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 (Hoang et al., 2018). A single transparent plaque was picked from the plate, suspended in SM buffer, incubated overnight at 4 °C, and passed through a 0.22-µm filter. The resulting filtrate was subjected to the above protocol three times in succession to purify the phage. The purified phage stock was prepared as described elsewhere (Hoang et al., 2018).

2.2 Transmission electron microscope examination

A highly concentrated suspension of phage (approximately 10^{10} 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.

2.3 One-step growth curve

The one-step growth curve of phage was established according to the method of Hoang et al. (2019), 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 (approximately 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 2,432 ×g for 5 min at 4 °C. The resulting pellet was suspended 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 ×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 (Hoang et al., 2019). The experiment was conducted in triplicate.

2.4 Challenge test in broth by single phage

The host bacterial culture in TSB was incubated at 30 °C with rotation at 40 rpm until its OD_{600} reached 0.1 (approximately 10^7 CFU mL^{-1}). The culture was then divided into two aliquots, of which, one was mixed with each of four phage lysates at a multiplicity of infection (MOI) of 2.0, and the other was not. The mixtures were incubated as described above, with samples being taken periodically for OD_{600} measurements. The experiment was conducted in triplicate.

2.5 Challenge test in broth by phage cocktail

The host bacterial culture was incubated at 30 °C, 120 rpm in TSB medium until its OD_{600} of 0.1 was attained (approximately 10^7 CFU mL^{-1}). The culture was divided into four aliquots. Each of three aliquots was mixed with each phage cocktail containing two different phages. MOI of each phage was 1.0 (a total MOI of two phages was 2.0). The mixtures were incubated as shown above. The fourth aliquot was left as a blank without phage addition. Each sample was withdrawn periodically and subjected to measure the OD_{600}. The experiment was conducted in triplicate.

3. Results and discussion

3.1 Isolation and morphology of phages

Four phages were isolated and purified from samples of striped catfish livers such as G1, G7, G8, and G9.2. These phages produced clear plaques with the diameters ranging from 1 to 8 mm (Figure 1). Stocks of four phages were then prepared with concentration of 10^{10} – 10^{11} PFU mL^{-1}. Based on the morphological analysis by transmission electron microscopy (Figure 1), all of isolated phages have icosahedral head with 46 – 48 nm

in diameter, and no tails. They were considered to be classified in the Corticoviridae or Tectiviridae family (Ackermann, 2009b).

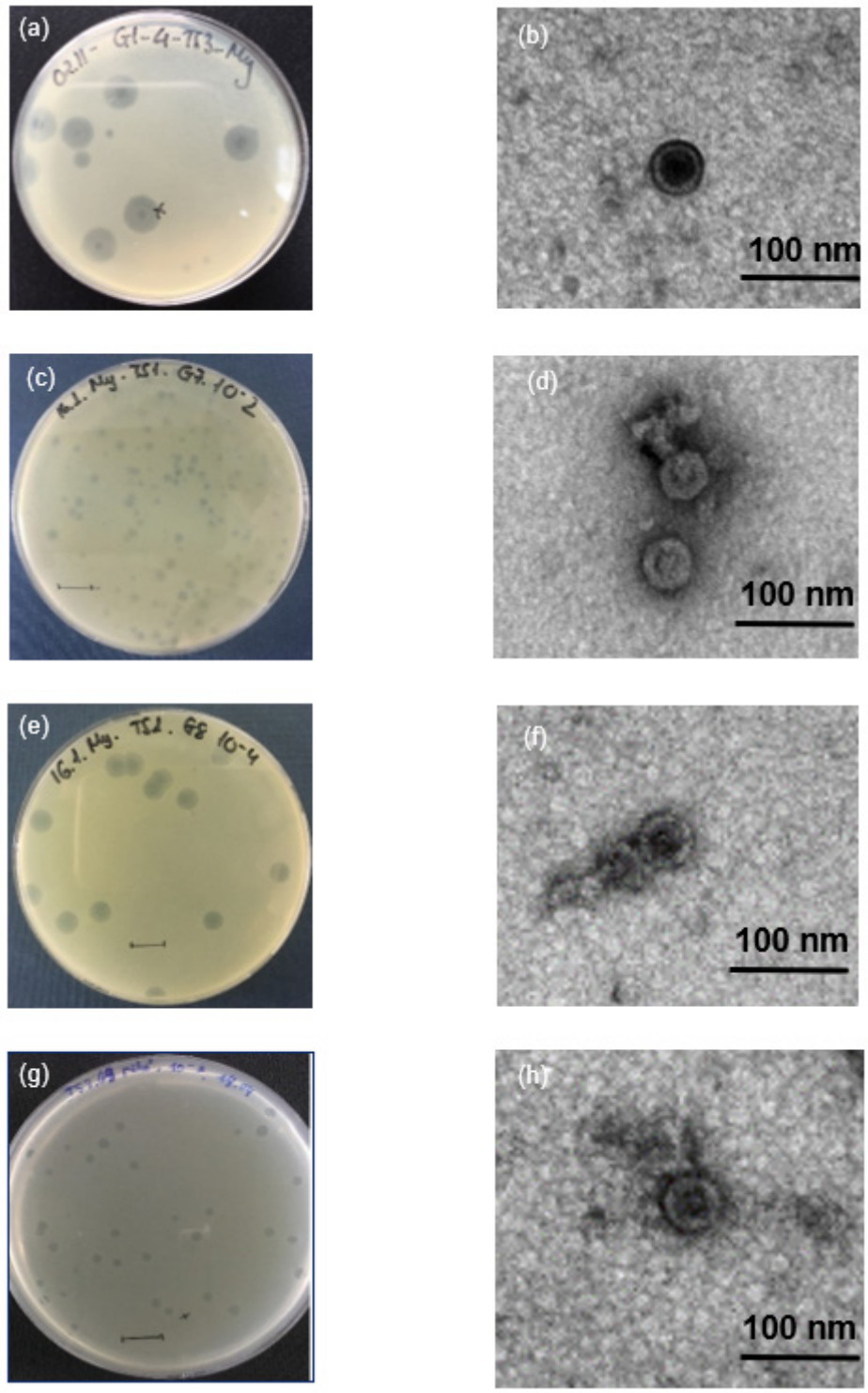

Figure 1: Phage plaques and respective electron micrograph of phages: phage G1 (a, b), phage G7 (c, d), phage G8 (e, f), phage G9.2 (g, h).

3.2 Latent period and burst size of phages

A phage is considered as highly lytic phage when it owns short latent period and/or large burst size. In this study, latent period and burst size of phage G1, G7, G8 and G9.2 were determined and shown in Table 1. Latent period of phage G1 was longer than that of G7 and G8 phages, but its burst size was the largest. The burst size of phage G1 was three times larger than phage MK7 isolated by Hoang et al. (2018). On the other hand, phage G7 had the shortest latent period with the second largest burst size.

Table 1: Lytic activity of the phages

Name of phage	Latent period (min)	Burst size (PFU/cell)
G1	70	160.52 ± 3.12
G7	55	71.70 ± 3.77
G8	60	28.22 ± 6.0
G9.2	70	41.72 ± 13.01

3.3 Inactivation of *E. ictaluri* by single phage

As shown in Figure 2.A, during the first h of incubation, increased OD_{600} were observed in the controls (*E. ictaluri* without G1, G7 or G8 phage), while no change in the OD_{600} of the mixture of *E. ictaluri* and G8 phage was observed. However, the OD_{600} of the mixture of *E. ictaluri* and G1 or G7 phage decreased sharply. After further incubation, the OD_{600} of all the bacterium-phage solutions decreased, while the OD_{600} of the controls increased. The transparency of bacterium-phage solutions (the OD_{600} were approximately lower than 0.1) was maintained for approximately 18 - 20 h indicating the efficacy of phage G1, G7 and G8 in inactivating the host cells. However, after approximately 20 h of incubation, the OD_{600} of the bacterium-phage solutions increased, reflecting the growth of phage-resistant bacteria.

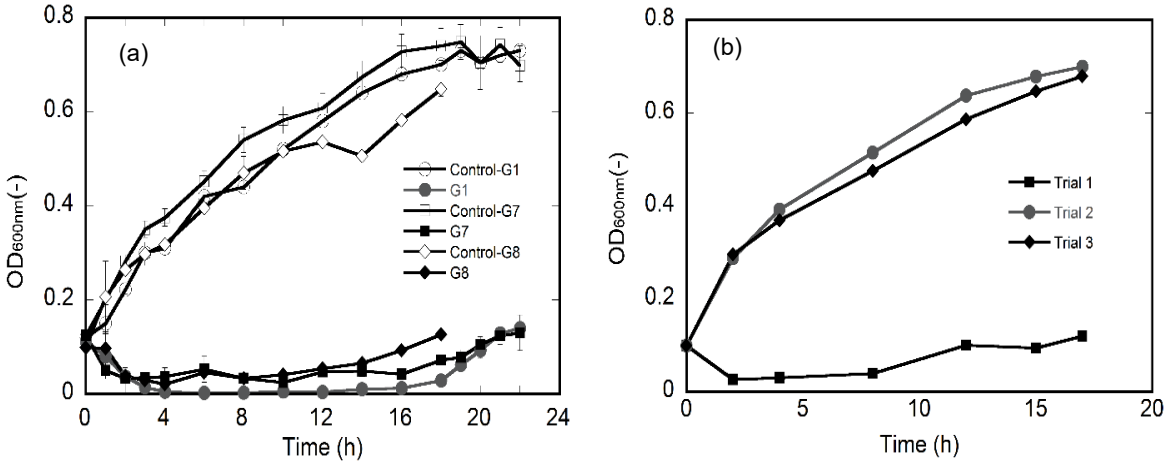

Figure 2: Changes in optical density at 600 nm during inactivation of E. ictaluri CT1 by phages. (a) G1, G7 or G8 phage; the negative control lacked phages. (b) G9.2 phage in three trials.

However, host cell suspensions treated by phage G9.2 resulted in very different time courses of the OD_{600} during three repeated trials (Figure 2.B). In the first trial, the OD_{600} change was similar to that in case of G1, G7 or G8 phage (Figure 2.A). However, in the second and third trials, the OD_{600} of the bacterium-phage solutions did not decrease. It indicated the efficiency of phage G9.2 in inactivating host cells was not stable. Chung et al. (2012) also showed similar results when testing the lytic activity of two temperate phages MP22 and D3112 to *Pseudomonas aeruginosa* PA14. The G9.2 phage was considered as a temperate phage. Temperate phages are generally not considered potential candidates for phage therapy (Philipson et al., 2018).

3.4 Inactivation of *E. ictaluri* by phage cocktails

The Figure 3 shows OD_{600} of the phage cocktail experiments. The OD_{600} of all the bacterium-phage solutions decreased and maintained lower than approximately 0.1 during the first 18 h. However, the OD_{600} of aliquots treated by the phage cocktails G1 + G7 or G1 + G8 sharply increased after approximately 18-h incubation. It was similar to the cases of single phage (Figure 2.A). Interestingly, in the case of phage cocktail G7 + G8, the OD_{600} remained lower than approximately 0.1 for 28-h incubation following by the fluctuation around 0.1. A clear

increase of the OD_{600} was observed only after approximately 45-h incubation. It indicated that the phage cocktail G7 + G8 was the most effective among the three phage cocktails in inactivation of the host cells.

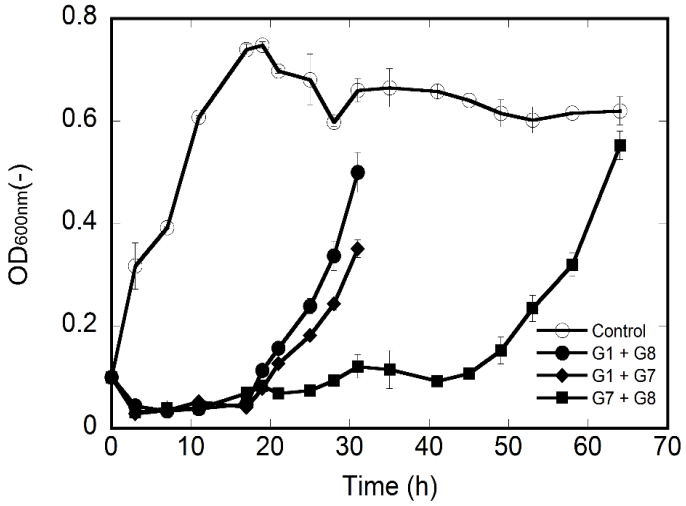

Figure 3: Changes in optical density at 600 nm during inactivation of E. ictaluri CT1 by phage cocktails; the negative control lacked phages.

The main concern of phage therapy needs to be considered is the regular appearance of phage-resistant bacteria (Culot et al., 2019). An effective approach to solve the problem is to apply phage cocktail to inactivate phage-resistant bacteria. A key factor for success of the phage cocktail is a collection of appropriate phages that infect host bacterium via attaching to different receptors on the bacterial cell wall. Filippov et al. (2011) showed that the phage cocktail can overcome the problem of the bacterial resistance only when the phages exploit different receptors. Similar observation was shown in research of Tanji et al. (2004) when cocktail of two phages can distinctly delay the appearance of phage-resistant cells. In research of Mateus et al. (2014), however, the efficiency of cocktail of three phages did not increase significantly compared to the use of single phage. It could be explained by the fact that all of the three phages might use the same bacterial receptor. In the current study, among three phage cocktails chosen, only the cocktail of G7 + G8 phages was markedly successful in inactivation of the host cells by delaying the appearance of phage-resistant cells. It might indicate that G7 and G8 phages infected the host bacterium via attaching to different receptors on the bacterial cell wall. The current study is the first report about success of phage cocktail to inactivate *E. ictaluri* in *in vitro* condition. In the future studies, the phage cocktail G7 + G8 will be investigated its capacity as potential agents to control *E. ictaluri* in striped catfish in the Mekong Delta, Vietnam.

4. Conclusions

Striped catfish is the most important farmed fish species in the Mekong Delta, Vietnam. However, white spots in the internal organs caused by *Edwardsiella ictaluri* is one of the most popular bacterial diseases that results in the decrease of striped catfish production efficiency in the region. Despite initial successes in using antibiotics as a measure of prevention and treatment, improper usage and inadequate control have led to the following alarming results such as output reduction and export loss, community health and environment degradation. This study introduced an effective approach such as phage cocktail as a replacement of antibiotics to control the pathogenic bacterium, *E. ictaluri*. Morphology, one-step growth curves, their latent period, burst size analysis of four phages were investigated. Inactivation of *E. ictaluri* by single phage and phage cocktail was also conducted. Among the three phage cocktails, the phage cocktail of G7 + G8 was markedly effective in inactivation of the host cells for 45 h (more than two folds than the other cases). This is the first report about success of phage cocktail to inactivate *E. ictaluri*, an infectious agent in striped catfish *Pangasianodon hypophthalmus*. In future studies, the protective efficacy of the phage cocktail against the disease in striped catifsh will be investigated.

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References

Ackermann H.W., 2009a, Basic Phage Electron Microscopy, Bacteriophages, Methods in Molecular Biology, 501, 103-116.

Ackermann H.W., 2009b, Phage Classification and Characterization, Bacteriophages, Methods in Molecular Biology, 501, 127-140.

Chung I.Y., Sim N., Cho Y.H., 2012, Antibacterial efficacy of temperate phage-mediated inhibition of bacterial group motilities, Antimicrobial Agents and Chemotherapy, 56(11), 5612–5617.

Culot A., Grosset N., Gautier M., 2020, Overcoming the challenges of phage therapy for industrial aquaculture: A review. Aquaculture, 513, 2020, 734851.

Dang T.H.O., Xuan T.T., Duyen L.T., Le N.P., Hoang H.A., 2021, Protective efficacy of phage PVN02 against haemorrhagic septicaemia in striped catfish Pangasianodon hypophthalmus via oral administration. Journal of Fish Diseases, 44(8), 1255–1263.

Doss J., Culbertson K., Hahn D., Camacho J., Barekzi N., 2017, A Review of Phage Therapy against Bacterial Pathogens of Aquatic and Terrestrial Organisms. Viruses, 9(3), 50.

Dong Z., Sha S., Li C., Hashim H., Gao Y., Ong P., Lee C.T., Zhang Z., Wu W.M., 2020, Potential Risk of Antibiotics Pollution in Aquaponic System and Control Approaches. Chemical Engineering Transactions, 78, 265–270.

Dung T.T., Crumlish M., Turnbull J.F., Ngoc N.T.N., Ferguson H.W., 2002, Identification of Edwardsiella ictaluri from diseased freshwater catfish, Pangasius hypophthalmus (Sauvage), cultured in the Mekong delta, VietNam. Journal of Fish Diseases, 25, 733–736.

Filippov A.A., Sergueev K.V., He Y., Huang X.Z., Gnade B.T., Mueller A.J., Fernandez-Prada C.M., Nikolich M.P., 2011, Bacteriophage-resistant mutants in Yersinia pestis: identification of phage receptors and attenuation for mice. PloS One, 6, e25486.

Hoang H.A., Yen M.H., Ngoan V.T., Nga L.P., Oanh D.T.H., 2018, Virulent bacteriophage of Edwardsiella ictaluri isolated from kidney and liver of striped catfish Pangasianodon hypophthalmus in Vietnam, Diseases of Aquatic Organisms, 132(1), 49–56.

Hoang H.A., Tran T.T.X., Le P.N., Dang T.H.O., 2019, Selection of phages to control Aeromonas hydrophila – an infectious agent in striped catfish, Biocontrol Science, 24, 23–28.

Kowalska J.D., Kazimierczak J., Sowińska P.M., Wójcik E.A., Siwicki A.K., Dastych J., 2020, Growing trend of fighting infections in aquaculture environment opportunities and challenges of phage therapy, Antibiotics, 9(6), 301.

Le T.S., Nguyen T.H., Vo H.P., Doan V.C., Nguyen H.L., Tran M.T., Tran T.T, Southgate P.C., Kurtböke D.İ, 2018, Protective effects of bacteriophages against Aeromonas hydrophila causing motile Aeromonas septicemia (MAS) in striped catfish, Antibiotics, 7(1), 16.

Mai T.T.N.L., Hoang H.A., Truong T.V., 2020, Antibacterial Activity of Tram Tron Syzygium Glomerulatum Extract against Methicillin-Resistant Staphylococcus Aureus, Chemical Engineering Transactions, 78, 235–240.

Mateus L., Costa L., Silva Y.J., Pereira C., Cunha A., Almeida A., 2014, Efficiency of phage cocktail in the inactivation of Vibrio in aquaculture. Aquaculture, 424, 167–173.

Philipson C.W., Voegtly L.J., Lueder M.R., Long K.A., Rice G.K., Frey K.G., Biswajit B., Regina Z.C., Theron H., Bishop-Lilly K.A., 2018, Characterizing Phage Genomes for Therapeutic Applications. Viruses, 10(4), 188.

Quach V.C.T., Tu T.D., Dang P.H.H., 2014, The current status antimicrobial resistance in Edwardsiellictaluri and Aeromonas hydrophila cause disease on the striped catfish farmed in the Mekong Delta, Journal of Science – Can Tho University, Special Issue: Aquaculture, 2, 7–14 (in Vietnamese).

Tanji Y., Shimada T., Yoichi M., Miyanaga K., Hori K., Unno H., 2004, Toward rational control of Escherichia coli O157: H7 by a phage cocktail, Applied Microbiology and Biotechnology, 64, 270–274.

VASEP, 2020, Challenges of export of striped catfish, Vietnam Association of Seafood Exporters and Producers <vasep.com.vn/san-pham-xuat-khau/ca-tra/nguyen-lieu/nhieu-chong-gai-cho-xuat-khau-ca-tra-20992.html> accessed 01.09.2021 (in Vietnamese).

Wu J.L., Lin H.M., Jan L., Hsu Y.L., Chang L.H., 1981, Biological control of fish bacterial pathogen, Aeromonas hydrophila, by bacteriophage AH 1. Fish Pathology, 15, 271–276.