Research Articles

Isolation and biological characterization of bacteriophages which infect *Aeromonas hydrophila*

Jiraporn Gatedee[1*] Sunsiree Muangman[1] Pornpan Pumirat[2] Yuvadee Mahakunkijcharoen[2] Noppadol Prasertsincharoen[3] Kanyanan Kritsiriwuthinan[1]

[1]Faculty of Medical Technology, Rangsit University
[2]Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University
[3]Department of Veterinary Technology, Faculty of Veterinary Technology, Kasetsart University

[*]Corresponding author, E-mail address: Jiraporn.g@rsu.ac.th

Abstract

This study aims to isolate and biological characterize bacteriophages which infect *Aeromonas hydrophila*, a causative of aquatic animal diseases. Two *A. hydrophila* bacteriophages, ØAH-S1 and ØAH-T5 were isolated from a total 100 water samples collected from ponds in Bangkok, Thailand. Electron micrograph indicated that these bacteriophages belonged to the *Myoviridae* family. Host-range determination revealed that ØAH-S1 and ØAH-T5 have different host range patterns and capable to lyse 62.0% (18/29) and 55.1 % (16/29) of tested *A. hydrophila*, respectively. The study on host-range among different species also showed different host range patterns and unable to lyse other tested bacteria including *Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Vibrio cholerae* and *Plesiomonas* spp. The efficiency to eradicate *A. hydrophila* ATCC 35654 *in vitro* showed that after 3 h of infection at multiplicity of infection 10, the reduction of average OD_{600} nm from both ØAH-S1and ØAH-T5 infected culture was decreased to 0.095 and 0.08 respectively compared with 1.679 of non-infected control. Therefore, this study demonstrated bacteriophages which infected *A. hydrophila* and these bacteriophages may be useful for future development as biocontrol agents for control of *A. hydrophila* infection in aqua animals.

Keywords: bacteriophage, *Aeromonas hydrophila*, characterization

การแยกและศึกษาคุณลักษณะทางชีวภาพของแบคเทอริโอฟาจของเชื้อ *Aeromonas hydrophila*

จิราภรณ์ เกตุดี[1*] สัณห์สิรี เมืองมาลย์[1] พรพรรณ ภูมิรัตน์[2] ยุวดี มหาคุณกิจเจริญ[2]
นพดล ประเสริฐสินเจริญ[3] กัญญนันทน์ กฤษศิริวุฒินันท์[1]

[1]คณะเทคนิคการแพทย์ มหาวิทยาลัยรังสิต
[2]ภาควิชาจุลชีววิทยาและอิมมิวโนโลยี คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล
[3]ภาควิชาเทคนิคการสัตวแพทย์ คณะเทคนิคการสัตวแพทย์ มหาวิทยาลัยเกษตรศาสตร์

[*]ผู้รับผิดชอบบทความ E-mail address: Jiraporn.g@rsu.ac.th

บทคัดย่อ

การวิจัยนี้ได้ทำการแยกและศึกษาคุณลักษณะทางชีวภาพของแบคเทอริโอฟาจของเชื้อซึ่งเป็นสาเหตุของโรคที่เกิดในสัตว์น้ำคือเชื้อ ***Aeromonas hydrophila*** วิธีการทดลองทำโดยเก็บตัวอย่าง 100 ตัวอย่าง จากแหล่งน้ำธรรมชาติ ในกรุงเทพมหานคร และสามารถแยกแบคเทอริโอฟาจของเชื้อ ***A. hydrophila*** ได้ 2 ไอโซเลท คือ ØAH-S1 และ ØAH-T5 เมื่อศึกษารูปร่างภายใต้กล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่านพบว่า ØAH-S1 และ ØAH-T5 จัดอยู่ใน family Myoviridae และเมื่อนำไปศึกษาโฮสต์-เรนจ์พบว่า ØAH-S1 และ ØAH-T5 มีโฮสต์-เรนจ์ต่างกันเมื่อทดสอบกับ ***A. hydrophila*** ที่แยกมาจากผู้ป่วย 29 ไอโซเลต โดยสามารถฆ่าเชื้อ ***A. hydrophila*** ที่แยกมาจากผู้ป่วยคิดเป็นร้อยละ 62.0 (18/29) และ 55.1 (16/29) ตามลำดับ และเมื่อนำมาทดสอบกับ Aeromonas สปีชีส์อื่นก็ยังแสดงผลโฮสต์-เรนจ์ต่างกัน อย่างไรก็ตาม ØAH-S1 และ ØAH-T5 ไม่สามารถฆ่าเชื้อแบคทีเรียแกรมลบชนิดอื่นที่นำมาใช้ในการทดสอบ เช่น ***Escherichia coli, Pseudomonas aeroginosa, Proteus mirabilis, Vibrio cholerae*** และ ***Plesiomonas*** spp. และเมื่อนำ ØAH-S1 และ ØAH-T5 มาทดสอบประสิทธิภาพในการฆ่าเชื้อโดยทำการกำจัดเชื้อ ***A. hydrophila*** ATCC 35654 ในหลอดทดลองพบว่า แบคเทอริโอฟาจทั้งสองชนิดสามารถทำให้เกิดปฏิกิริยา clear lysis ภายในเวลา 3 ชั่วโมง ที่ MOI = 10 โดยพบว่าค่าเฉลี่ย OD_{600} nm. ของ ØAH-S1 และ ØAH-T5 มีค่าลดลงเป็น 0.095และ 0.080 ตามลำดับ ในขณะที่หลอดที่ไม่ใส่แบคเทอริโอฟาจ ให้ค่าเฉลี่ย OD_{600} นาโนเมตร เท่ากับ 1.679 ดังนั้นในการศึกษาครั้งนี้ได้นำเสนอคุณสมบัติของ ØAH-S1 และ ØAH-T5 และข้อมูลที่ได้อาจใช้เป็นข้อมูลที่สำคัญสำหรับการพัฒนาควบคุมติดเชื้อ ***A. hydrophila*** ในอุตสาหกรรมสัตว์น้ำต่อไป

คำสำคัญ : แบคเทอริโอฟาจ *Aeromonas hydrophila* การศึกษาคุณลักษณะ

Introduction

A. hydrophila is a Gram-negative bacilli that cause motile Aeromonad septicemia in human, aquatic animals, amphibians and reptiles (Bi et al. 2007). The bacteria can be found from environmental such as water, fish, domesticated pets and natural soils (Janda JM and Abbott SL. 2010). It is one of the most important agents of the outbreaks in aquaculture (El-Araby et al. 2016). In 2012, Shayo SD et al. described that *A. hydrophila* is an important causative agent of ulcerative diseases in fish from dam at Tanzania (Shayo et al. 2012). Recently, the outbreak has also occurred in the southeastern United States which resulting in industry-wide losses of channel catfish (Hossain MJ. et al. 2014). Now a day, due to the improper use of antibiotics by fisher folk, the emergence of *A. hydrophila* resistance antibiotic is typically common in aquaculture (Cabello FC. 2006). Therefore, it needs to find the new approach to control *A. hydrophila* infection.

Bacteriophages or phages are viruses that specific to kill bacteria. They are the most abundant and generally can be isolated from the environments that are habitats for the bacterial host such as soil, deep sea vents, water and sewage (Wommack KE and Colwell RR. 2000). Bacteriophage can be either temperate where they are integrated into the genomes of their host bacteria or lytic where they infect, multiply and kill their host (Campbell A. 2003). This property of lytic bacteriophages makes them attractive as an alternative for therapeutic agent for combat bacterial disease. Several studies reported the effectiveness of using bacteriophages for the treatment of bacterial diseases in aqua animals (Karunasagar I et al. 2005; Donna MA et al. 2016) and for controlling bacterial populations in water (Jun JW et al. 2016). However, the data of *A. hydrophila* bacteriophages in Thailand has not been described. Therefore, this study tends to isolate and biological characterize of *A. hydrophila* bacteriophages from several water sample in Bangkok. The isolated bacteriophages could further be used as biocontrol agents for control of *A. hydrophila* infection.

Materials and methods

Bacterial strains

A. hydrophila ATCC 35654 was used as indicator strain for bacteriophages isolation. In addition, *Aeromonas hydrophila, Aeromonas trota, Aeromonas sobia, Aeromonas media, Aeromonas jandaei, Aeromonas caviae, Aeromonas veronii, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Vibrio parahaemolyticus, Vibrio cholerae* and *Plesiomonas* spp. were included for bacterial host specificity test.

Collection of water samples

Water samples were collected from natural ponds in public parks in Bangkok, Thailand during May-June, 2013. Water samples were collected with 10-20 cm in depth and kept in sterile containers.

Bacteriophage isolation and purification

Bacteriophage enrichment, isolation and purification were performed as previously described (Van Twest R et al. 2009; Kropinski AM et al. 2009).

Bacteriophage enrichment

An overnight culture of *A. hydrophila* ATCC 35654 was inoculated into the mixture of equal volume of water sample and 2 X TSB and incubated at 37°C for 16-18 h. Then, the mixture was centrifuged at 10,000 x g for 10 min. The supernatant was filtered through 0.45 μm filter into sterile tubes and kept at 4°C until use.

Spot test

Spot test was performed for screening of *A. hydrophila* bacteriophages. Ten microliters of filtrated samples prepared from 3.3.1 was drop onto $1x10^8$ cfu/ml of A. hydrophila ATCC 35654 cultures which spread onto TSA supplemented with CaCl2. After that, plates were incubated at 37°C for 16-18 h, a clear zone in the spotted area (putative *A. hydrophila* bacteriophage) was selected for confirmation by double agar plaque assay.

Double agar plaque assay

Two hundred microliters of putative *A. hydrophila* bacteriophage was mixed with 100 µl of *A. hydrophila* ATCC 35654 (OD_{600}=0.20) and incubated at 37°C for 15 min to allow bacteriophage adsorption. Then the mixture was added into overlay media (0.4% TSA containing CaCl2) and poured on TSA plate containing CaCl2. The plaque colonies were observed after overnight incubation at 37°C

Bacteriophage purification

A. hydrophila lytic bacteriophages were purified by picking up a single plaque using a sterile yellow tip. The plug was suspended in 1 ml of SM buffer (50 mM Tris-Cl, pH 7.5, 99 mM NaCl, 8 mM MgSO4, 0.01% gelatin) and left at 4°C overnight to allow free bacteriophages suspended in the solution. Then, the putative bacteriophage suspension was serially diluted and subjected to double agar plaque assay at least three times to ensure a clonal selection.

Biological Characterization

Transmission electron microscopy (TEM)

Bacteriophages morphology was determined by TEM at Center of Nanoimaging, Mahidol University, Thailand. Briefly, purified bacteriophage concentration 10^9 pfu/ml was negatively stained by 1% uranyl acetate and viewed on TECNAI 20 TWIN microscope.

Bacterial host range

The susceptibility of newly isolated bacteriophages was determined with *Aeromonas* spp. and other tested bacteria such as *E. coli, P. aeruginosa, P. mirabilis, V. parahaemolyticus, V. cholerae* and *Plesiomonas* spp. using spot test. Ten microliter of bacteriophage at concentration of 10^8 pfu/ml was drop onto bacterial host culture previously spread onto TSA supplemented with CaCl2. After that, plates were incubated at 37°C for 16-18 h, a clear zone in the spotted area indicated a positive result.

The efficiency to eradicate the host bacterium *in vitro*

The efficiency to eradicate the host bacterium in vitro was determine as previous described (Yordpratum U et al. 2010). Briefly, the host bacterium was performed by growing *A. hydrophila* ATCC 35654 in TSB with shaking at 37°C. After bacterial growth until OD_{600} = 0.2 (Ca. 10^8 pfu/ml), bacteriophage solution was added at multiplicity of infection (MOI) 0.1, 1.0 and 10. The numbers of bacteria in bacteriophage infected solution and non-infected control were measured an hour interval using spectrophotometer at 600 nm for 8 h. The tests were determined in duplicate and the average OD of bacteriophage infected solution and non-infected controls were plot in graph.

Results

From screening of 100 water samples by spot test, two samples showed clear zones on lawn of *A. hydrophila* ATCC 35654, suggesting the positive bacteriophages isolation. Source of these positive isolates were shown in Table 1. After confirmed with plaque assay, both of them generate clear plaques with diameter approximately 2 mm. After more than 3 rounds of bacteriophages purification, 2 isolated bacteriophages were purified and designated as ØAH-S1 and ØAH-T5. TEM showed that ØAH-S1 and ØAH-T5 had icosahedral capsid with diameter ~50 nm and ~60 nm respectively and the long tail with sheath which are typical morphology of bacteriophages in the *Myoviridae* family (Fig. 1).

Host- range determination revealed that ØAH-S1 and ØAH-T5 have different host range patterns and capable to lyse *A. hydrophila* clinical isolated samples from human 62.0% (18/29) and 55.1 % (16/29), respectively. The study on host-range among different *Aeromonas* species showed that both bacteriophages able to lyse *A. trota, A. sobia, A. media, A. caviae, A. veronii*. However, only ØAH-T5 is able to lyse *A. jandaei*, suggesting that ØAH-T5 has different host range patterns with ØAH-S1. In addition, both bacteriophages unable to lyse other tested bacteria including *E. coli*, *Pseudomonas aeruginosa, Proteus mirabilis, Vibrio cholerae* and *Plesiomonas* spp. suggesting the bacteriophages, ØAH-S1 and ØAH-T5 specific to infect *Aeromonas* spp. (Table 2).

Table 1 The location of water samples collection, the number of isolated *A. hydrophila* bacteriophages, and name of bacteriophages

Location	Number of water samples	Number of isolated *A. hydrophila* bacteriophages	Name
1. Lumphini Park	50	1	ØAH-S1
2. Vachirabenjatas Park	50	1	ØAH-T5

ØAH-S1and ØAH-T5 showed the efficiency to eradicate the host bacterium *in vitro* by clear lysis of infected bacteria at MOI of 0.1, 1.0 and 1.0. Interestingly, after 3 h of infection at MOI of 10, the reduction of average OD_{600} nm from both ØAH-S1and ØAH-T5 infected culture was observed to nearly zero, 0.095 and 0.08 respectively, while uninfected control was 1.679 (Fig. 2).

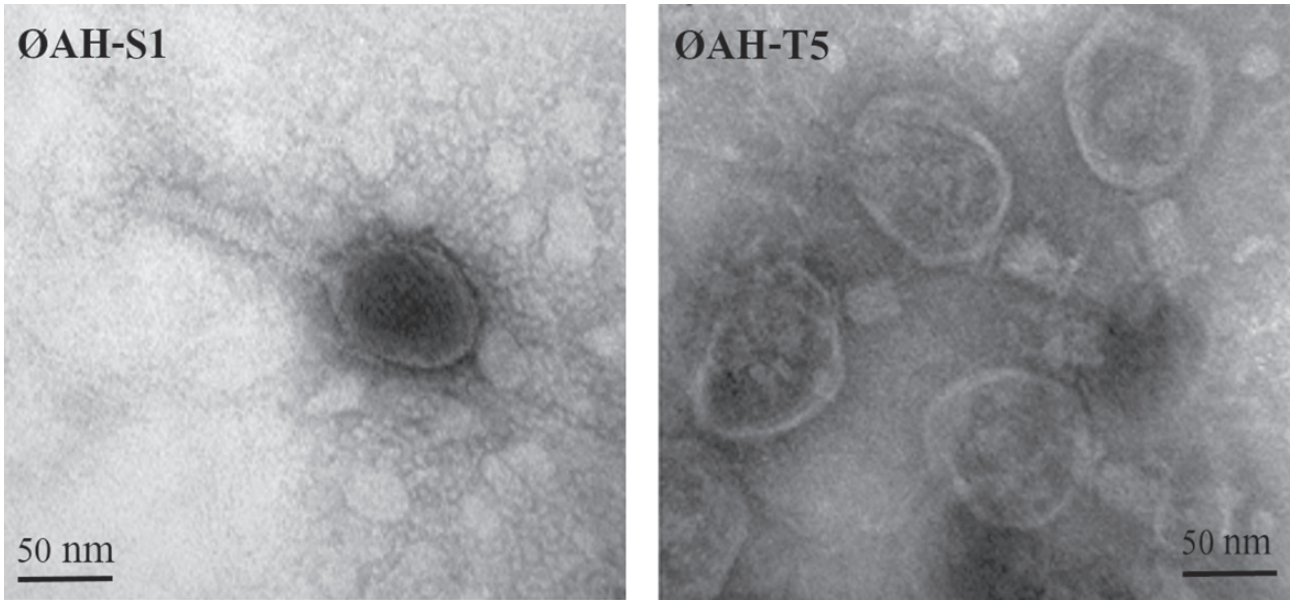

Figure 1. Transmission electron micrograph analysis of ØAH-S1 and ØAH-T5. Bar 50 nm.

Table 2. Host range of ØAH-S1 and ØAH-T5 on *A. hydrophilla, Aeromonas* spp. and other bacterial species by spot test

Bacteria	Source	Number of isolates	Percentage of infection	
			ØAH-S1	ØAH-T5
A. hydrophila	Environmental isolation	3	100	100
A. hydrophila	Clinical isolation	29	62.0	55.1
A. trota	Clinical isolation	6	83.3	83.3
A. sobia	Clinical isolation	7	14. 3	14.3
A. media	Clinical isolation	3	66.7	66.7
A. jandaei	Clinical isolation	4	0	75
A. caviae	Clinical isolation	5	100	100
A. veronii	Clinical isolation	4	25	25
E. coli	ATCC 25992	1	0	0
E. coli	Clinical isolation	10	0	0
P. aeruginosa	Clinical isolation	5	0	0
P. mirabilis	Clinical isolation	5	0	0
V. cholerae	Clinical isolation	1	0	0
Plesiomonas spp.	Clinical isolation	1	0	0

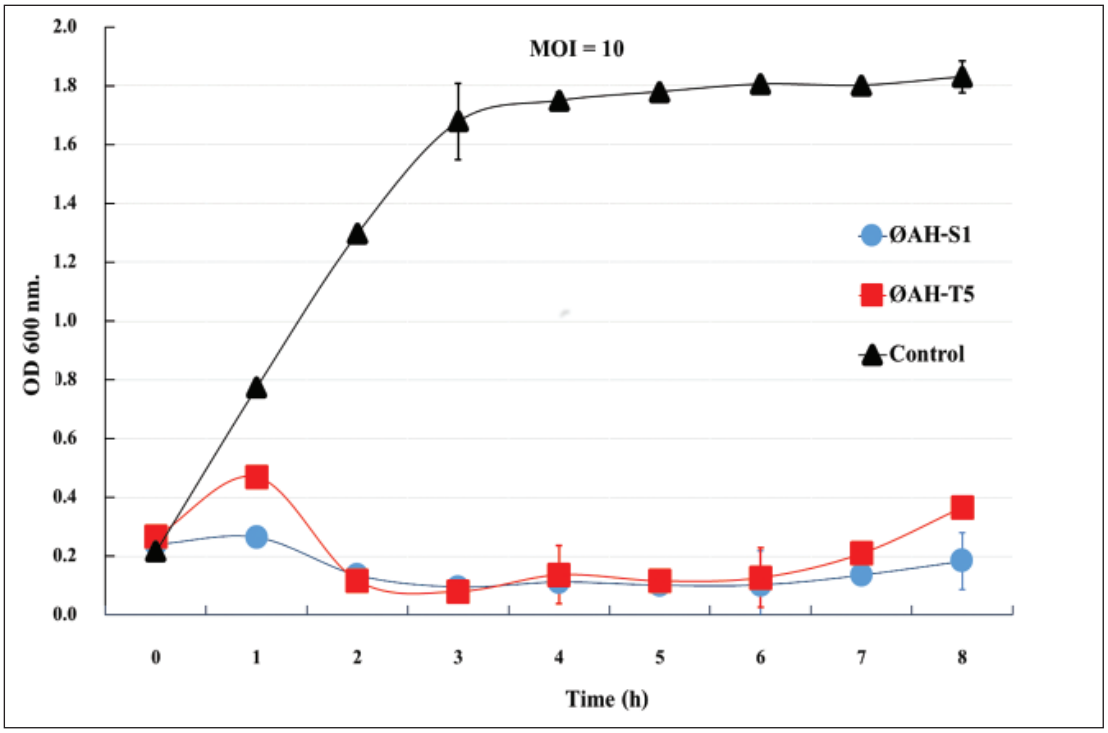

Figure 2. The efficiency to eradicate the host bacterium *in vitro* of ØAH-S1 and ØAH-T5 at MOI 10. The reduction of *A. hydrophila* ATCC 35654 in ØAH-S1 (●) or ØAH-T5 (■) infected and non-infected control (▲) were determined at OD_{600} nm. All of the tests were performed in duplicate.

Discussion

A. hydrophila was described as the dominant infectious agent of aqua animals bacterial septicemia especially fish in freshwater aquaculture (Samal KS et al. 2014). As *A. hydrophila* typically resistance to most common antibiotics, control of *A. hydrophila* infection is becoming more difficult. Therefore, the alternative ways should be developed and applied to control of *A. hydrophila* infection in aquaculture.

In this study, *A. hydrophila* bacteriophages, ØAH-S1 and ØH-T5 were isolated from freshwater at the parks in Bangkok, Thailand. These bacteriophages, ØAH-S1 and ØAH-T5 are belonging to the Myoviridae family which similar to the previous isolated *A. hydrophial* bacteriophage isolated from an organized equine farm (Taruna Anand et al. 2016). Host- range determination revealed that ØAH-S1 and ØAH-T5 have the efficiency to lyse *A. hydrophila* both clinical and environmental isolates. They also infect and lyse the closely related strain such as *A. trota, A. sobia, A. media, A. caviae, A. veronii* which is similar to the previously reported (Easwaran M et al. 2016), suggesting both bacteriophages could be applied to control multiple strains of *Aeromonas*. However, only ØAH-T5 is able to lyse *A. jandaei*, suggesting that it not identical to ØAH-S1. However, the molecular characteristics different of both bacteriophages should be verified by restriction endonuclease patterns of the digest genomic DNA or DNA sequence analysis.

ØAH-S1and ØAH-T5 showed the efficiency to eradicate the host bacterium *in vitro* by clear lysis at MOI of 0.1, 1.0 and 1.0. Interestingly, after 3 h of infection at MOI 10 show markedly reduction of

OD_{600} nm of both ØAH-S1and ØAH-T5 infected culture to nearly zero. However, after 6 and 7 h of infection, the numbers of *A. hydrophila* ATCC 35654 in ØAH-S1and ØAH-T5 infected culture was increased again. This characteristic of bacteriophage infection was also demonstrated in *Burkhoderia pseudomallei* (Yordpratum U et al. 2010. The possible explanation for the presence of incomplete lysis might be due to 1) the bacterial cell debris containing bacteriophage receptors that could block bacteriophages multiplication 2) ØAH-S1and ØAH-T5 could integrate into *A. hydrophila* ATCC 35654 and transforming to the lysogenized cells which in turn activated to superinfection by the same bacteriophage or 3) the number of bacteriophages added to bacterial cultures is not enough to kill large number of bacterial host cells. However, further experiment should be investigated to elucidate the protective effects of phage therapy against fish pathogenic *A. hydrophila* in aqua animal's model.

Conclusion

This study shows the isolation of a *Myoviridae* family bacteriophages which infect *A. hydrophila*. Both bacteriophages, ØAH-S1 and ØAH-T5 have different host range patterns to infected bacteria. The efficiency to eradicate the host bacterium *in vitro* demonstrated the efficiency of ØAH-S1and ØAH-T5 at MOI of 10 to kill *A. hydrophila* ATCC 35654 *in vitro* suggesting these bacteriophages may be useful for further development as biocontrol agents for control of *A. hydrophila* infection in aquatic farm.

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