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感染潰瘍病細菌 *Edwardsiella tarda* 之新噬菌體株 ϕET-1 之研究

Isolation and Application of a New Bacteriophage, ϕET-1, Which Infect *Edwardsiella tarda*, the Pathogen of Edwardsiellosis

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Abstract

The first bacteriophage which infect and lyse *Edwardsiella tarda*, the pathogen of fish edwardsiellosis, was isolated from one of the 350 screened water samples and was named as ϕET-1. Bacteriophage ϕET-1 had wide spectrum of host range by showing 92.6% of the virulence in 27 strains of *E. tarda*. Bacteriophage ϕET-1 had strong killing power for *E. tarda* by its quick lysis ability. The viable *E. tarda* could be reduced to less than 0.1% of the starting concentration by ϕET-1 infection at an M. O. I.=0.08 in 8 hours. In the meantime, ϕET-1 phage were under active multiplication of infective viral particles. Immersion of loaches *Misgurnus anguillicaudatus* in *E. tarda* suspension rather than injection was chosen for the assessment of biological control measure of ϕET-1. The pathogenecity of *E. tarda* was almost completely eliminated after 8 hours by ϕET-1 infection at an M. O. I.=0.1. The shorter time of infection (2 or 4 hours) and lower M. O. I. (0.01, 0.001, 0.0001) of infection by ϕET-1, the pathogenecities of *E. tarda* were partially retained. By the above data, the biological control of *E. tarda* by ϕET-1 is feasible.

Introduction

Edwardsiellosis is caused by the infection of *Edwardsiella tarda* and is one of the most common fish diseases in the culture pond[5,16]. *E. tarda* had been isolated from eel[10,11,16], channel catfish[6,15], goldfish[9] and large mouth bass[17]. The infected eels show ecchymoses and petechiae on the body surface, mainly on the belly surface, putrefactive lesions of the liver and kidney, and the necrosis of the skin and muscle[8,10,11]. The worldwide distribution of *E. tarda* and high mortality caused by *E. tarda* had drawn the intensive study and practice of prevention and therapy of edwardsiellosis. At present, the drugs such as antibiotics, nitrofurans and sulfonamides are widely used in the hatcheries[7,12]. However, due to the poor ingestion of the diseased fish, the deposition of drug residues and the induction of resistant strains of *E.*

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tarda, the chemotherapeutic method is not successful for edwardsiellosis in the long term practice of chemical drugs.

The virulent bacteriophages can specifically infect the host bacteria and result in cell lysis within one or several hours[1]. After one phage growth cycle, several hundreds of phage progenies are multiplicated from one phage-infected bacterium. By the specific properties of bacteriophage, the pathogenic bacteria can be controlled at low concentration by phage infection and reach the purpose of specific biological control of fish diseases[20]. However, no any known virulent bacteriophage which could infect *E. tarda* as host cell had been reported. In this paper, we described the first successful isolation of bacteriophage ϕET-1 which could infect *E. tarda*. The assessment of the efficacy of the biological control of edwardsiellosis by this bacteriophage was also presented.

Materials and Methods

1. Bacteria

Different strains of *Edwardsiella tarda* were isolated from diseased fishes in different locus of Taiwan. Those strains were identified and obtained from Department of Zoology, National Taiwan University and Department of Veterinary Medicine, National Ping-Tung Agricultural College.

2. Media

(i) 3XD medium[19]: 3XD medium was prepared by mixing sterilized solution A (9 gm of KH_2PO_4, 21 gm of Na_2HPO_4, 30 gm of casein hydrolysate, 26 gm of glycerol and 60 mg of gelatin in 1,900 ml of H_2O) and sterilized solution B (0.6 gm of $MgSO_4 \cdot 7H_2O$ and 0.6 ml of 1 M $CaCl_2$ in 100 ml of H_2O).

(ii) Diluting medium: 1,000 ml of H_2O contained 0.8 gm of nutrient broth and 5 gm of NaCl.

(iii) Bottom agar medium: It was prepared by dissolving 10 gm of Bactoagar, 13 gm of Bactotryptone, 8 gm of NaCl, 2 gm of sodium citrate$\cdot H_2O$ and 1.3 gm of glucose in 1,000 ml of H_2O.

(iv) Soft agar medium: This medium was same as Bottom agar medium except Bactoagar and glucose were reduced to 6.5 gm and 3 gm, respectively.

3. Isolation of Bacteriophage

The pond and sewerage waters were collected from different locus. The sample water was centrifuged at 15,000×g for 10 minutes at 4°C to sediment the removable contaminants. Then the supernatant was filtered through the millipore filter (0.22 μm). The bacteriophages contained in the filtrate were multiplicated by enrichment culture in *E. tarda*[1]. 3 ml of the filtrate was added to 1 ml of the early *log* phase of *E. tarda*. After 24 hours of aerobic cultivation at 28°C, the bacterial debris were removed by centrifugation and the supernatant was assayed for the presence of bacteriophages by plaque forming method[1] The bacteriophage plaque was removed from agar surface and subjected to infect *E. tarda* culture in liquid medium.

4. Infection of ϕET-1

The oevrnight culture of *E. tarda* was diluted 100 times in 3XD medium and aerated at

28°C. When the bacteria grew to $OD_{450nm}=1.0$, the culture was centrifuged and the pellet was suspended in sterilized tap water to a concentration of 1.1×10^{9} cells per ml. The *E. tarda* suspension was infected with ϕET-1 at an M. O. I. (multiplicity of infection)=0.1. The viable *E. tarda* and ϕET-1 changes were followed by colony formation and plaque-forming, respectively.

5. ***E. tarda*** **Pathogenecity of Test**

The pathogenecity test was performed by injection of *E. tarda* suspension to loach, *Misgurnus anguillicaudatus*, or immersion of loaches in *E. tarda* suspension. For the injection methods, the loach was injected with *E. tarda* at a dosage of 4×10^{7} cells per gm of body weight by ventral or dorsal injection. For the immersion method, the loach was immersed in 1×10^{8} cells per ml or lower concentration of *E. tarda* suspension for one hour. The pathogenecity and mortality were observed following the treatments.

Results and Discussion

Growth Curve of *Edwardsiella tarda*

The outbreak of edwardsiellosis was observed during the end of spring when the water temperature reached around 25°C with fluctuation[2,10,11]. By this character, *E. tarda* was considered as warm-water fish pathogen. For the isolation and cultivation of *E. tarda*, it is usually grow on Rimler-Shotts medium (R-S medium) of Trypticase Soy medium (T-S medium)[11]. In this paper, *E. tarda* was grown in 3XD medium or nutrient broth at 28°C for the infection and multiplication of bacteriophage. In order to understand the growth curve of *E. tarda* under our condition, the *E. tarda* overnight culture was diluted 100 times with 3XD medium and cultivated at 28°C with aeration. As shown in Fig. 1, the first two hours was the *lag* phase which showed almost no viable cell increase. Between two to seven hours after subculture, the bacteria division went rapiply and the cell number increased from 1.5×10^{6} cells/ml to 9×10^{8} cells/ml. This rapid increase period belongs to *log* phase and is the best host cells for viral infection and growth[20]. After *log* phase, the cell growth rate was slowed down and went to stationary phase. In the mean time, the absorbancy of the culture was measured at $\lambda=450$ nm and expressed as O. D. value (Fig. 1). For example, $OD_{450nm}=1.0$ will correspond to 1×10^{8} cells/ml in *E. tarda* culture, the suitable infection condition by bacteriophages can be determined.

Isolation of Bacteriophage ϕET-1

E. tarda strain A-49 was used as host for the enrichment culture and plaque-forming of bacteriophage isolation. From the 350 water samples, one bacteriophage was isolated and named as ϕET-1. ϕET-1 is a virulent bacteriophage by having a property of strong cell-lysis property[14]. As indicated in Table 1, ϕET-1 had wide host range of infection. 92.6% (25/27) of the *E. tarda* strains were susceptible to ϕET-1 infection either by plating efficiency or lysis spot test. The plating efficiency of ϕET-1 could be ranged between 3.9% and 176.3% in different host strains by comparing with strain AT-49 (100%). The usefulness of this bacteriophage in biological control purpose depends on the host range of infection[20]. The wider host

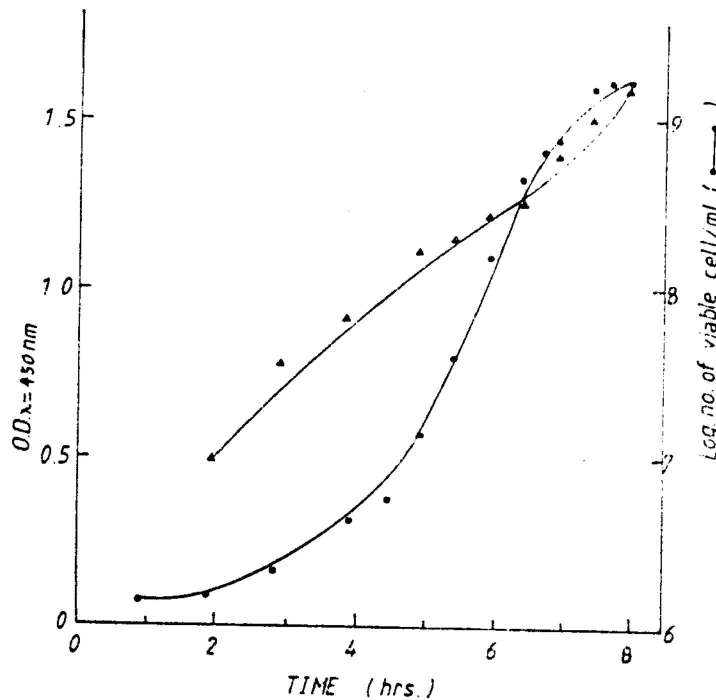

Fig. 1. Growth curve of *Edwardsiella tarda* at 28°C with aeration.
•---• Viable cells; ▲---▲ OD_{450} nm.

Table 1. Host range of bacteriophage ϕET-1 infection of *Edwardsiella tarda*

Strains	Titer (×10⁷/ml)	Plating efficiency (%)	Strains	Titer (×10⁷/ml)	Plating efficiency (%)
800312-1L	402	176.3	810217-1I	44	19.3
810312-1K	392	171.9	AK-301	24	10.5
AT-49	228	100	800129-1Ib	23	10.1
AT-53	204	89.5	AK 300	9	3.9
800325-6L	175	76.7	AC 60	+	
810217-2I	152	66.7	AC 54	+	
AT-44	138	60.5	760508-3SK	+	
800423-2K	126	55.2	800123-5L	+	
810424-4K	119	52.2	800323-6L	+	
800312-1I	119	52.2	800129-5L	+	
AT-46	118	51.7	810217-1L	+	
800325-6K	95	41.6	AW-286	−	
AT-58	67	29.3	800423-1L	−	
810217-21	56	24.6			

Note: +: cell lysis; −: no cell lysis

range of infection will simplify the application of bacteriophage. By this criteria, bacteriophage ϕET-1 has a great potential for the study of biological control of edwardsiellosis by having lysis ability and wide host spectrum of infection.

Killing of *E. tarda* and Growth of ϕET-1

The overnight culture of *E. tarda* was diluted and grew in 3XD medium at 28°C. When

the bacteria grew to $OD_{450nm}=1.0$, then the cells were pelleted by centrifugation and suspended in sterilized tap water to a concentration of 1.2×10^9 cells/ml. This bacteria suspension was infected with ϕET-1 at an M.O.I.=0.08. The viable *E. tarda* concentration was reduced immediately following the bacteriophage infection (Fig. 2). The survival bacteria was less than 0.1% of the starting concentration after 8 hours of infection. After longer time of ϕET-1 infection, very low percentage of survival *E. tarda* at a same reduction rate can be expected. In the mean time, the plaque-forming units of bacteriophage ϕET-1 were increased from 1×10^8/ml to 1×10^9/ml. The ϕET-1 progenies could infect the survived *E. tarda* for further replication. Therefore, the drastic killing of *E. tarda* to very low concentration and the continuous growth of ϕET-1 can happened in the water system. Due to active replication of ϕET-1, the practice of this method for disease control will be economic and feasible.

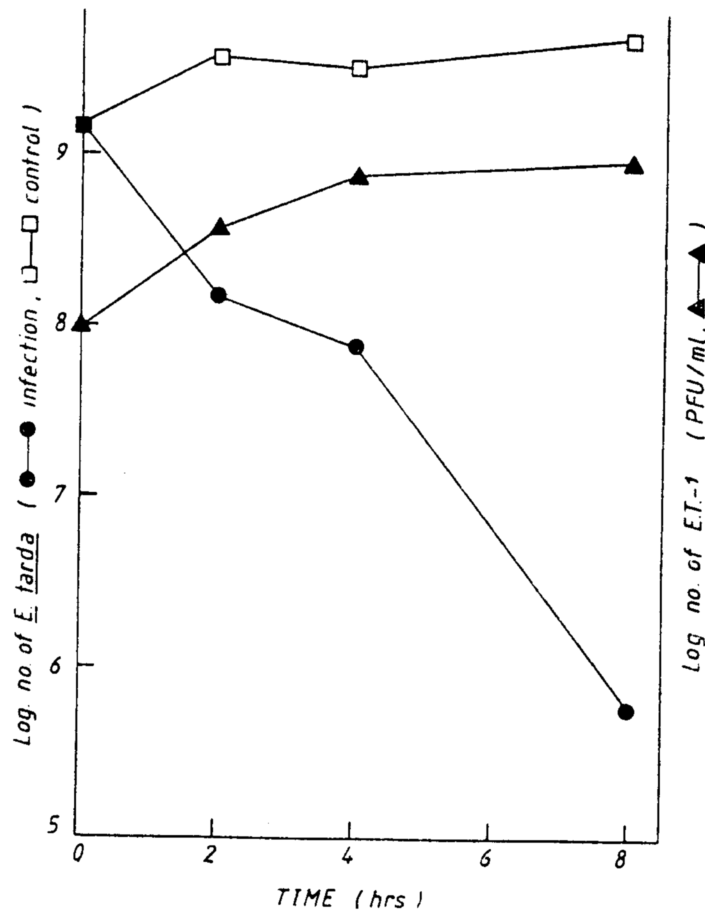

Fig. 2. Killing of *Edwardsiella tarda* and multiplication of bacteriophage ϕET-1.

Selection of Pathogenecity Test

The selection of a simple and rapid measure for *E. tarda* application to loach, *Misgurnus anguillicaudatus*, will benefit to the assessment of the control efficacy of ϕET-1. Usually, the dorsal injection is the often used method to perform *E. tarda* pathogenecity test by observing inflammation, necrosis, and mortality[10]. In this study, the survivals of the loaches after ventral injection, dorsal injection or immersion of *E. tarda* were compared (Fig. 3). The immersion method had quickest killing effect on the treated loaches. During the one hour im-

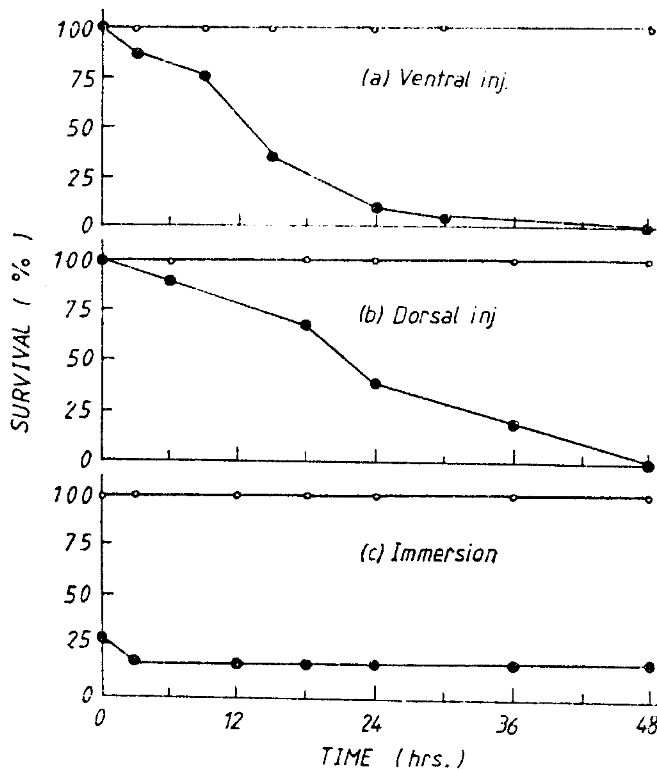

Fig. 3. Pathogenecity test of *Edwardsiella tarda* by ventral injection, dorsal injection or immersion of loaches *Misgurnus anguillicaudatus*.
•—• Treated; ○—○ Control.

mersion in 1×10^8 cells/ml of *E. tarda*, more than 70% of the loaches were infected and killed. This lethal effect might be due to quick infection through gills in high concentration of *E. tarda* and caused the blockage of respiration. The ventral injection or dorsal injection had relatively slower lethal effect. All the loaches were infected and killed at 48 hours after infection. In the mean time, the inflammation and necrosis could be observed on the body surface. By the immediate effect, the immersion method was the best choice for the assay for pathogen elimination by ϕET-1.

The loaches were immersed in different concentrations of *E. tarda* and to justify the pathogen dosage effect. As shown in Fig. 4, the loaches could stand at 1×10^7 or 1×10^6 cells per ml of *E. tarda* for more than 48 hours without any mortality. The critical concentration of *E. tarda* to express pathogenic effect will between 1×10^7 to 1×10^8 cells per ml. The immersion concentration of pathogen at 1×10^8 cells/ml was the choice for the following biological control studies.

Elimination of *E. tarda* Pathogenecity by ϕET-1

The *log* phase culture of *E. tarda* was centrifuged and resuspended in sterilized tap water to a concentration of 1×10^8 cells per ml for bacteriophage ϕET-1 infection at an M. O. I. of 0.1. After different times of ϕET-1 infection, the loaches were immersed in the bacteriophage-infected pathogen suspension for one hour and the mortality was observed. After 8 hours of ϕET-1 infection, over 90% of the loaches could survive in the bacteriophage-infected suspension for more than 4 days (Fig. 5). 2 and 4 hours post-infection of ϕET-1, the survival percentages

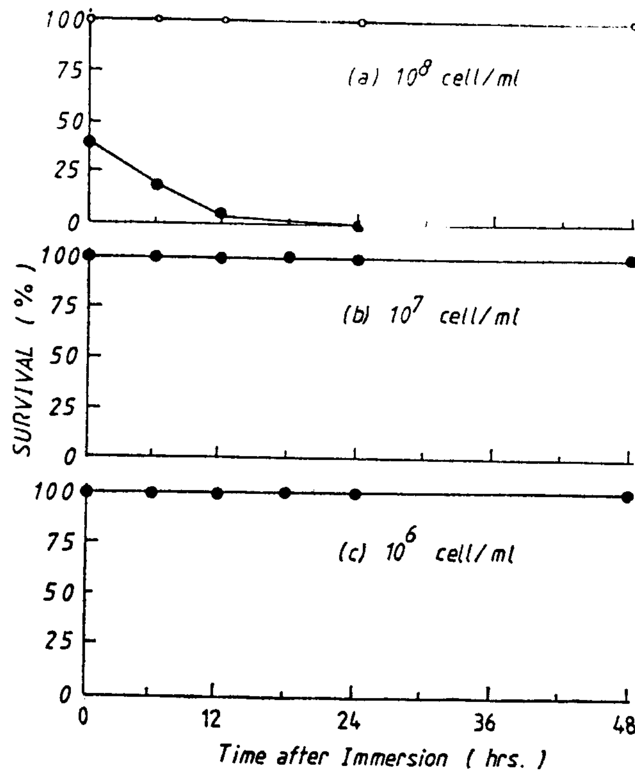

Fig. 4. Pathogenic effect of *Edwardsiella tarda* on loached under different immersion concentrations.

•—• Treated; ○—○ Control.

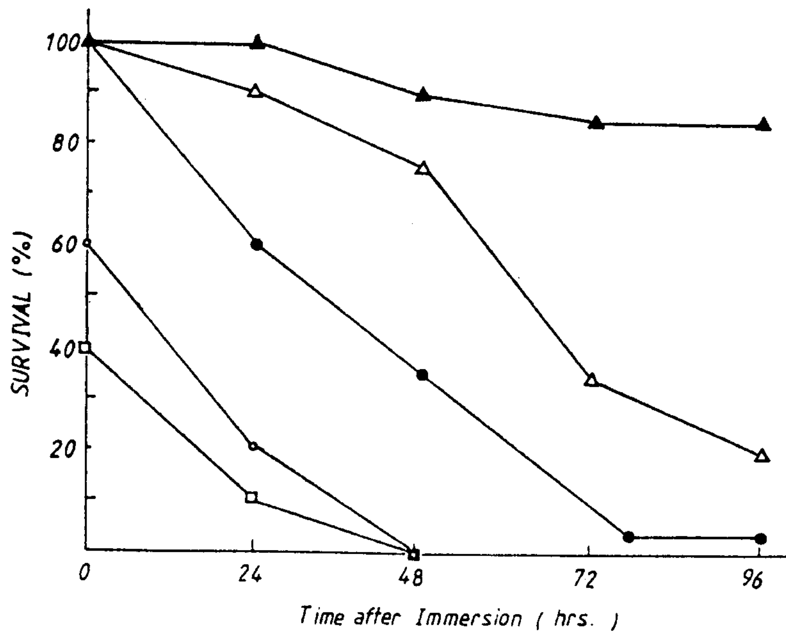

Fig. 5. Elimination of the pathogenecity of *Edwardsiella tarda* after bacteriophage ϕET-1 infection with different times. The M. O. I. was 0.1 and the immersion time of loaches was 1 hour.

▲—▲ 8 hours; △—△ 4 hours; •—• 2 hours; ○—○ 2 minutes; □—□ Uninfected *E. tarda*.

of loaches were reduced to 5% and 20% in the same period of immersion. The immediate exposure of loaches to *E. tarda* following ϕET-1 infection for 2 minutes had similar killing effect as the uninfected *E. tarda* group which showed complete mortality of fishes within 48 hours. The different ability of ϕET-1 to eliminate *E. tarda* pathogenecity was related to the time of infection; the longer time post ϕET-1 infection resulted in better protection of fishes. This phenomenon was the reflection of the multiplication character of bacteriophage[4,18]. The complete growth cycle of bacteriophage consists of adsorption to host cell surface, replication of nucleic acid, assembly of virion and release of bacteriophage progenies[3,13]. The total time required for the above process is usually finished within one or two hours at 30°C. The 2 minutes ϕET-1 infected *E. tarda* suspension still contained 90% or more of the alive bacteria, therefore, the pathogenecity was showed up as quick as uninfected control group. On the other hand, after 8 hours of ϕET-1 infection, the bacteriophage had opportunity to multiplicate for several cycles and the *E. tarda* bacteria were almost lysed by this long time infection. The result was the loaches could survive under this condition. However, 2 or 4 hours infection of ϕET-1 was not long enough to have complete killing of *E. tarda*, thus the partial pathogenic effect was retained.

The different M. O. I. of ϕET-1 to infect *E. tarda* for 8 hours and the pathogenic effects to loaches were followed (Fig. 6). At an M. O. I.=0.1 or 1.0, the elimination of pathogenecity was very successful. When the M. O. I. was reduced to 0.01, 0.001 or 0.0001, only 30%, 25% or 10% of the loaches could survived at 96 hours after immersion. The partial pathogenecity of low M. O. I.-infected *E. tarda* was due to the 8 hours infection of ϕET-1 was too short for

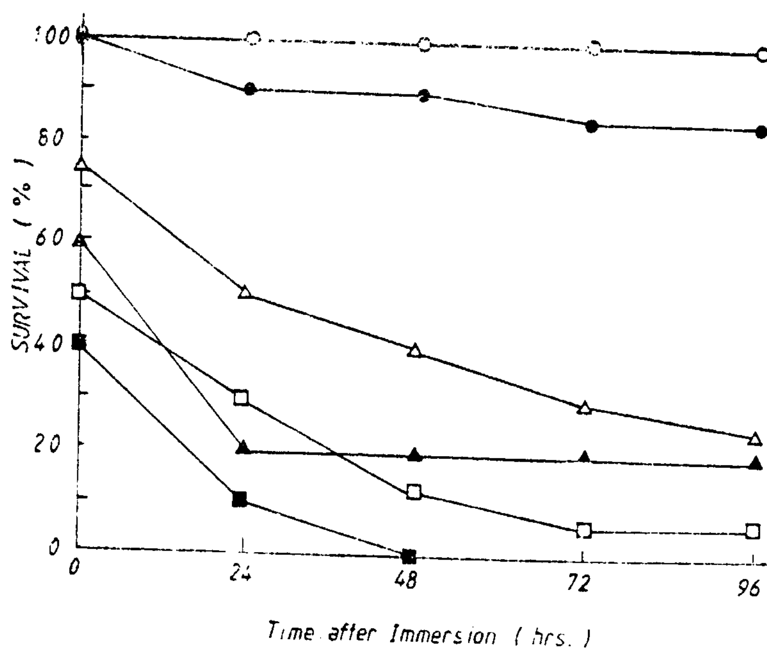

Fig. 6. Effect of the multiplicity of infection (M. O. I.) of bacteriophage ϕET-1 on the pathogenecity of *Edwardsiella tarda*. *E. tarda* was infected by ϕET-1 for 8 hours and the loached were immersed for 1 hour in this test.
○—○ M. O. I.=1.0; •—• M. O. I.=0.1; △—△ M. O. I.=0.01; ▲—▲ M. O. I.=0.001; □—□ M. O. I.=0.0001; ■—■ Uninfected *E. tarda*.

enough bacteriophage multiplication. But for long term infection, the low M. O. I. was applicable to control *E. tarda* at low concentration.

Bacteriophage AH1 had selected as a biological control agent for fish red-fin disease by infecting and killing *Aeromonas hydrophila*[20]. However, bacteriophage AH1 had a rather limited range of infection. In this study, bacteriophage ϕET-1 had wide spectrum of infection, rapid killing on the host cells, continuous replication of bacteriophages and effective elimination of *E. tarda* pathogenecity, therefore, this bacteriophage could be considered as a powerful and potential biological control agent for *E. tarda* in pond water. Further studies such as the replication conditions of bacteriophage ϕET-1, the stabilities of ϕET-1 under different environment and the scale-up of the control measure pond should be justified before the application of this bacteriophage in cultured ponds.

中 文 摘 要

由350個池水或下水道之水樣品中，分離出第一株能感染潰瘍病細菌 *Edwardsiella tarda* 及溶菌能力之噬菌體，它被命名爲 ϕET-1。噬菌體 ϕET-1 具有廣泛的寄主感染範圍，27株 *E. tarda* 菌株中的25株會受到 ϕET-1 之感染而溶菌。*E. tarda* 菌液經過感染倍率 (multiplicity of infection) 爲0.08之噬菌體 ϕET-1 感染8時後，活菌量僅爲原來數目的 0.1%以下；同時，可以測出 ϕET-1 會進行快速的增殖生長。將泥鰍 *Misgurnus anguillicaudatus* 浸浴於 *E. tarda* 菌液中，觀察活存魚體之數目來評估噬菌體之生物防治的價值；在噬菌體感染倍率爲0.1來感染 *E. tarda*，經過8小時後，細菌之病原性幾乎完全被消除，但是，若時間更短(2或4小時)及低感染倍率 (0.01, 0.001 或 0.0001)時，則部份之 *E. tarda* 病原性仍然保留下來。由本研究之結果看來，噬菌體 ϕET-1 應用於潰瘍病之生物防治乃爲可行之道。

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