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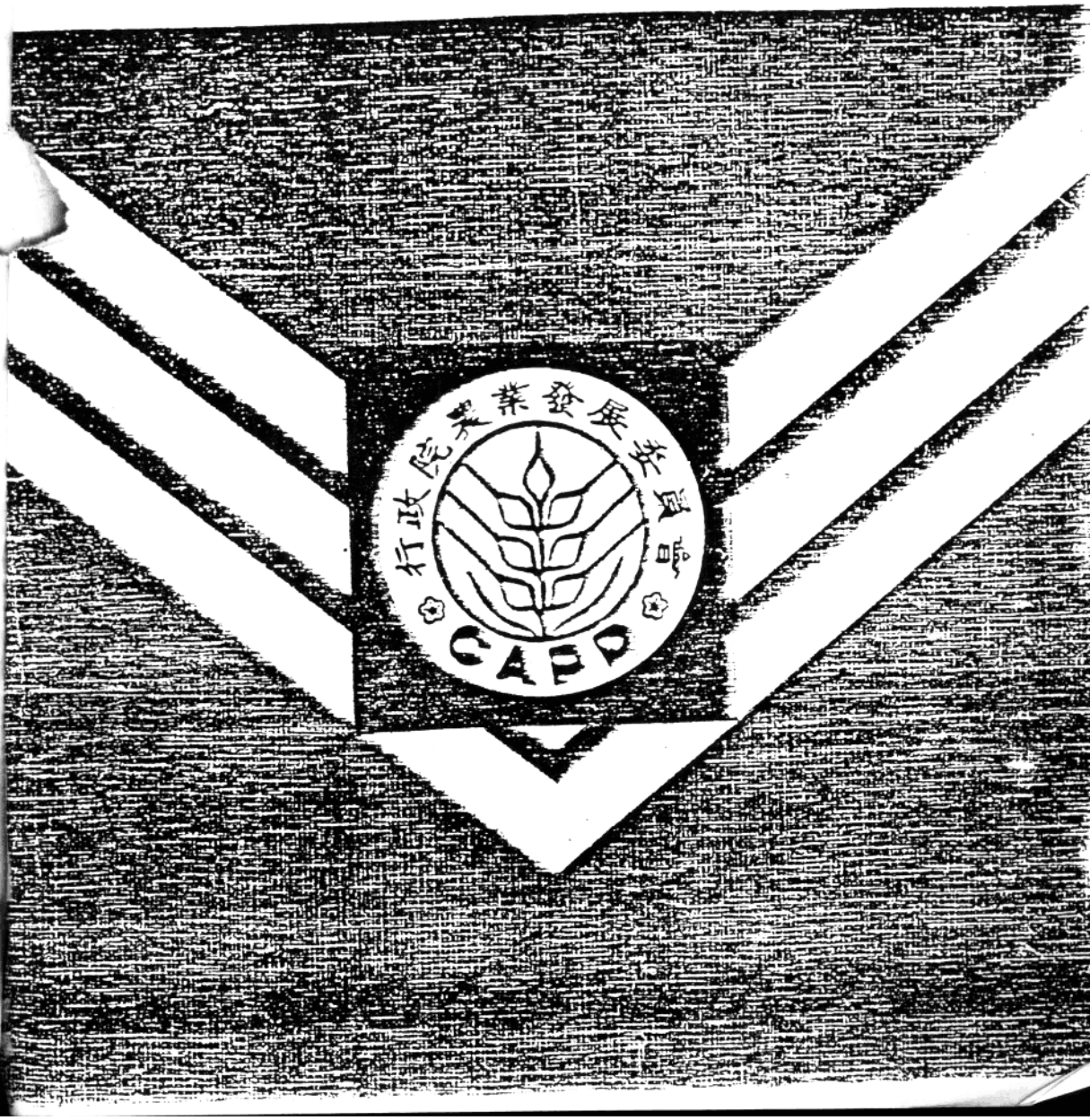
農發會漁業特刊第八號
CAPD Fisheries Series No.8

魚病研究專集(四)

Reports on Fish Disease Research (Ⅳ)



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日本鰻魚之腎臟及卵巢細胞株的雙向擴散及免疫電泳研究

Immunodiffusion and Immunoelectrophoresis Studies of Cell Lines Derived from Japanese Eel (*Anguilla japonica*) Ovary and Kidney

陳秀男・郭光雄

Shiu-Nan Chen and Guang-Hsiung Kou*

Abstract

The application of immunodiffusion and immunoelectrophoresis for distinguishing fish cell lines was evaluated in the present study. The results showed that eel cell lines derived from kidney and ovary tissues revealed cross-reactive precipitin lines with either heterogeneous or homogeneous cell lines. But, the number of precipitin lines resolved in the immunoelectrophoresis plates could be used as criteria for the identification of eel cell lines at a familic level.

By using immunodiffusion and immunoelectrophoresis techniques common cross-reactive antigens between eel cell lines and homogeneous tissues were consistently observed, but they were absent in the heterogeneous systems.

Introduction

Up to the present more than 60 fish cell lines have been established and they were derived from various tissues of approximately 36 species of fish (Wolf and Mann, 1980). Concurrent with the increased number of available lines, there is an urgently need for criteria by which either the cell origin can be identified or one line can be distinguished from the others.

It was demonstrated that cell morphology, growth characteristics, karyologic pattern could be used as parameters for the characterization of animal cell lines from different taxonomic orders. Moreover, immunologic and enzymatic techniques were also suggested to be useful in the identification of cell lines derived from different orders.

Although several mammalian or invertebrate cell lines have been reported to be contaminated by extraneous cells (Fogh, 1973; Greene and Charney, 1971; Greene *et al.*, 1972), Aldridge and Knudson (1980) could distinguish five lepidopteran cell lines by using immunoelectrophoresis. These results may reflect that immunoelectrophoresis is useful in the identification of cell line with different origin.

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In the present study, the valuation of immunodiffusion and immunoelectrophoresis for the characterization of eel ovary and kidney cell lines (Chen and Kou, 1981; Chen *et al.*, 1982) was detected.

Materials and Methods

Cell Lines

Four fish cell lines including EK-1, EO-2, EPC and RTG-2 and one mammalian cell line, HeLa were used in the present study. All the fish cell lines were cultured in Leibovitz's L-15 medium supplemented with 10% foetal calf serum, 400 Units/ml penicillin, 400 μg/ml streptomycin and 20 μg/ml fungizone. HeLa cells were maintained in Eagle's minimal essential medium (MEM) supplemented with the identical substances as described above. All the tissue culture media and supplements were obtained from GIBCO, New York, USA.

Preparation of Immunizing Antigens

The cell lines grown on 75 cm^2 Falcon flask surface were harvested by centrifugation and followed by washing the cells several times with Hank's balanced salt solution (HBSS). The cell pellet was then re-washed with HBSS for four times to eradicate the substances in the culture media. The final pellet was suspended in a little amount of distilled water and homogenized with 0.5 cc Bellco glass homogenizer. The supernatant was then stored in deep freezer (−70°C) until experimental uses.

Preparation of Antisera

Antisera against EK-1 and EO-2 cell antigens were prepared in two rabbits weighing 2-3 kg. Each rabbit received four weekly subcutaneous inoculation with antigens in an amount of 5, 7.5, 10, 15 mg protein respectively. Prior to the immunization, each antigen was homogenized with complete Freund's adjuvant (Difco) in a ratio of 1:1 (v/v). At the seventh day after the final booster injection, the immunized rabbits were bled by cutting carotid artery and the blood were collected. Antisera were then obtained by centrifugation of the blood at 6,000×g for 10 minutes. The complete Freund's adjuvant was also injected into a rabbit for 4 times weekly. Antisera obtained from this rabbit were used as control.

Preparation of Test Antigen

Cultured cell line and tissue extract antigens were used in the present study.

For the preparation of cell line antigens, the cell lines described above were grown on the surface of 75 cm^2 Falcon flasks and cells were harvested and washed four times in HBSS. The final pellet was extracted using the sucrose-acetone extraction procedures of Clarke and Casals (1958).

Tissue extracts were obtained by homogenizing of tissues from ovary, kidney, spleen and heart of Japanese eel or Common carp respectively. Prior to the homogenization, the individual tissue removed from the fish was washed five times to eradicate the blood. The tissue was then homogenized with 15 cc Ballco homogenizer. Protein concentrations of the preparation were estimated by using the Folin-phenol method (Lowry *et al.*, 1951).

Immunodiffusion and Immunoelectrophoresis

1.2% Agarose (w/v) in veronal buffer (diethylbarbituric acid, 1.4 gm; sodium diethylbarbiturate, 5.0 gm; sodium chloride, 1.0 gm; distilled water 1 liter), pH 8.4, was used for both immunodiffusion and immunoelectrophoresis. Sodium azide was added into the agarose at a final concentration of 0.02% (w/v) to prevent microbal growth.

In immunodiffusion tests, a circular pattern of walls around central well were made on 8×8 cm agar slides, the center-to-center distance from the wells being 1 cm. Each well was 4 mm in diameter. The central wells were filled with antisera and peripheral wells were filled with antigens.

Immunoelectrophoresis was also performed on 8×8 cm agar slides and serum trough (2× 67 mm) was kept 4 mm, edge to edge. The experiment was then carried out at approximately 10°C under 10 mA/Plate constant current.

For both experiments 500 μg protein of antigen was added to each well and antisera were used undiluted.

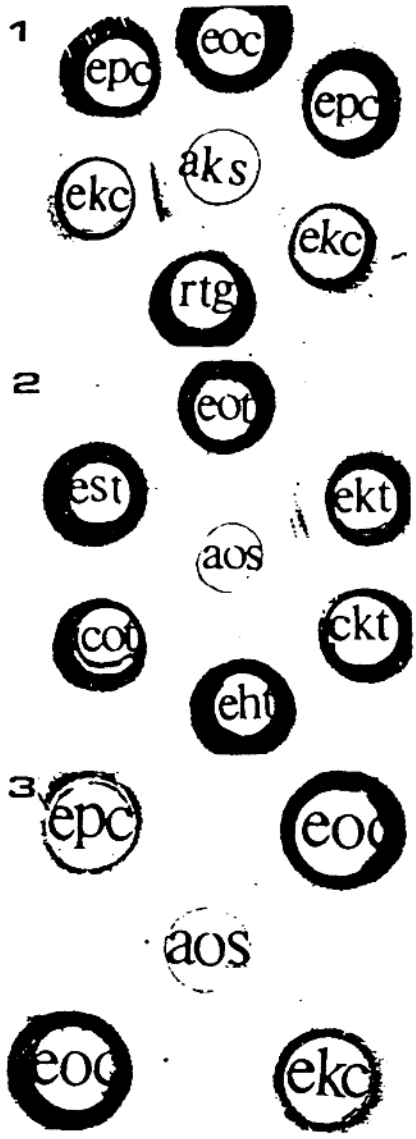
The immunodiffusion and immunoelectrophoresis plates were incubated at 37°C for 48 hours and prepared for staining by washing the plates in three changes of normal saline with a final rinse in distilled water. The agarose was then air dried by covering of filter paper to the plates, stained in 0.1% (w/v) Amindo Black 10 B in 5% (v/v) acetic acid and destained in 7% (v/v) acetic acid.

Results

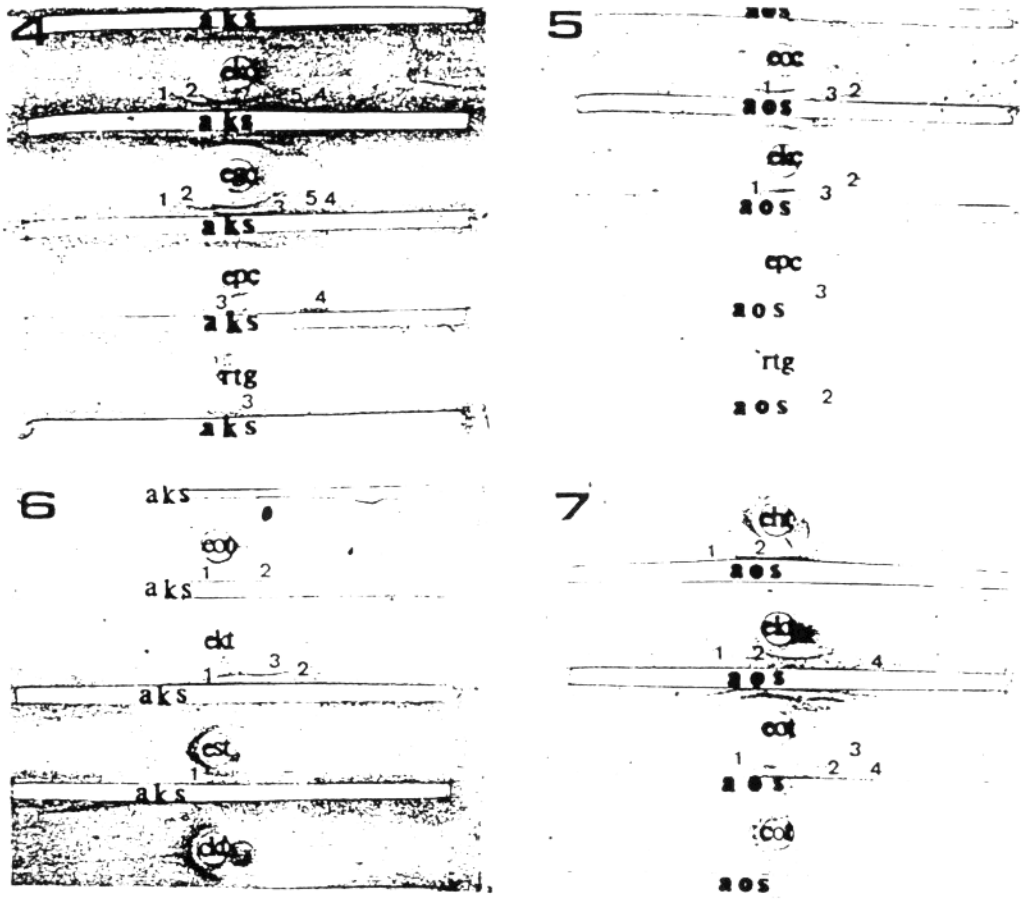
Using immunodiffusion and immunoelectrophoresis no precipitin line was observed between anti-Freund's adjuvant serum and EK-1 or EO-2 cell line antigen. Similarly, the negative result was also obtained when anti-sera against EK-1 and EO-2 cells reacted with mammalian cell line, HeLa.

The immunodiffusion demonstrated that anti-EK-1 and anti-EO-2 sera reacted with all the tested fish cell line antigens (Figs. 1 and 3). Best resolution of precipitin lines was usually observed in homologous reactions. Strong reactions were also observed when anti-EK-1 or anti-EO-2 serum rected with the tested antigens originated from eel kidney or ovary, respectively. However, no precipitin line was observed, when carp ovary and kidney antigens reacted with antiserum against EO-2 (Fig. 2).

Reciprocal immunoelectrophoresis test demonstrated that EK-1 and EO-2 cell lines were cross-reactive with the tested fish cell lines including EPC and RTG-2 (Figs. 4 and 5). Table 1 showed the number of precipitin lines resulted in immunoelectrophoresis plates. The results demonstrated that when anti-EK-1 serum reacted with EO-2 or EK-1 antigen respectively, the similar number and pattern of precipitin lines were observed. The identical results were also obtained when anti-EO-2 serum reacting with EK-1 or EO-2 antigen was performed. However, less precipitin lines were observed when anti-EK-1 or anti-EO-2 serum reacted with EPC or RTG-2 antigen, respectively. Immunoelectrophoresis plates of antigens originated from eel and common carp tissues relative to anti-EK-1 or anti-EO-2 serum were presented in Figs. 6 and 7. The results showed that various eel tissue antigens exhibited cross-reactive precipiting antigen



Figs. 1–3. Photomicrographs representing the image of fixed, stained immunodiffusion plates in reactions of anti-eel cell line (anti-EK-1 or anti-EO-2) sera with various antigenic complexes. aks: Anti-EK-1, aos: Anti-EO-2, eoc: EO-2 cell line antigen, ekc: EK-1 cell line antigen, epc: EPC cell line antigen, rtg: RTG-2 cell line antigen, eot: Eel ovary antigen, est: Eel spleen antigen, ekt: Eel kidney antigen, eht: Eel heart antigen, cot: common carp ovary antigen, ckt: common carp kidney antigen.



Figs. 4–7. Photomicrographs representing the image of fixed, stained immunoelectrophores plates in reactions of anti-eel cell line (anti-EK-1 or anti-EO-2) sera with various antigenic complexes. aks: Anti-EK-1, aos: Anti-EO-2, eoc: EO-2 cell line antigen, ekc: EK-1 cell line antigen, epc: EPC cell line antigen, rtg: RTG-2 cell line antigen, eot: Eel ovary antigen, est: Eel spleen antigen, ekt: Eel kidney antigen, eht: Eel heart antigen, cot: common carp ovary antigen, ckt: common carp kidney antigen.

(s) common to the tested eel cell lines (Table 1). No cross-reactive precipitin arcs were found when the antigens originated from carp reacted with antisera against EK-1 or EO-2 cell lines (Figs. 6 and 7; Table 1).

Discussion

Although immunoelectrophoresis is demonstrated to be useful in detecting similarities and differences amongst different molecules, EK-1 and EO-2 cell lines derived from tissues of Japanese eel (*Anguilla japonica*) were not differentiated using this approach. From the presence

Table I. Number of precipitin lines resolved in reactions between given antisera and antigens by using immunoelectrophoresis

Antisera	Antigens Derived from	No. of Precipitin Lines
Anti-EK-1	EK-1	5
	EO-2	5
	EPC	2
	RTG-2	1
	Eel Kidney	3
	Eel Ovary	2
	Eel Spleen	1
	Common Carp Kidney	0
Anti-EO-2	EK-1	3
	EO-2	3
	EPC	1
	RTG-2	1
	Eel Kidney	3
	Eel Ovary	4
	Eel Heart	2
	Common Carp Ovary	0

EK-1: Japanese eel (*Anguilla japonica*) kidney cell line EO-2: Japanese eel (*Anguilla japonica*) ovary cell line. EPC: Epithelioma papillosum cyprini tissue cell line; a cell line from *Cyprinus carpio*. RTG-2: Rainbow trout (*Salmo gairdneri*) gonad cell line.

of precipitin patterns, these two cell lines were distinguished from the other two cell lines which derived from the fishes belonging to families Cyprinidae and Salmonidae, suggesting that the serologic approach involving in the present study allowed distinction to be made at familic level. In the present study, the differentiation of fish cell lines was only performed by comparing the different degree of cross-reactivity of antigens. The perfect differentiation could be resulted from the availability of high specificity of antisera. The cross-adsorption of sera may provide a greater specific in the antisera, but, this is very time consumming and required a greater expenditure of effort. It is therefore, suggested that either immunodiffusion or immunoelectrophoresis is not ideal technique for the differentiation of fish cell lines with different origin. The presence of detective cross-reactive antigen between different cell lines may reflect the limitation in the application of immunodiffusion and immunoelectrophoresis on the characterization of fish cell lines.

The presence of precipitin lines between antisera against eel cell lines and eel tissues demonstrated the cell lines still possess their original property. In comparison, there were no common cross-reactive nature was obtained between eel cell lines and common carp tissues.

In the study of lepidopteran cell lines, Aldridge and Knudson (1980) demonstrated that differentiation of these cell lines at a familic level was possible by using serological techniques including complement fixation, hemagglutination, immunodiffusion and immunoelectrophoresis. They also suggested that immunoelectrophoresis was the best amongst the four used serological techniques for distinguishing lepidopteran cell lines. Similarly, the present study also demonstrated that the immunoelectrophoresis is better than immunodiffusion in the differentiation of

fish cell lines.

Apart from the serological techniques, the other approach, isozyme analyses, was reported to be superior to the serological techniques for the characterization of insect cell lines at an intrageneric level (Green, 1971; Greene *et al.*, 1972; Tabachnick and Knudson, 1980). In our laboratory, experiments are in progress which, it is hoped, will evaluate the application of isozyme analyses on the identification of fish cell lines.

中 文 摘 要

本研究乃利用雙向擴散及免疫電泳法，來評估此兩種方法應用於魚類細胞株之區別的可能性。實驗結果顯示，抗鰻魚卵巢及腎臟細胞株之免疫血清與其他魚類細胞株抗原有交叉反應作用。但由免疫電泳實驗所呈現之沈澱線數目，可判別同科或非同科之魚類細胞株。

又，利用雙向擴散及免疫電泳法亦證實了鰻魚細胞株與鰻魚組織抽取液間有共同抗原之存在。但若以鯉魚組織抗原與抗鰻魚細胞株血清作用等，則未有任何沈澱線出現。

Acknowledgments

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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

吳 金 洌 · 趙 偉 眞

Jen-Leih Wu and Wei-Jen Chao

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[6,18], 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[6,10,11]. 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

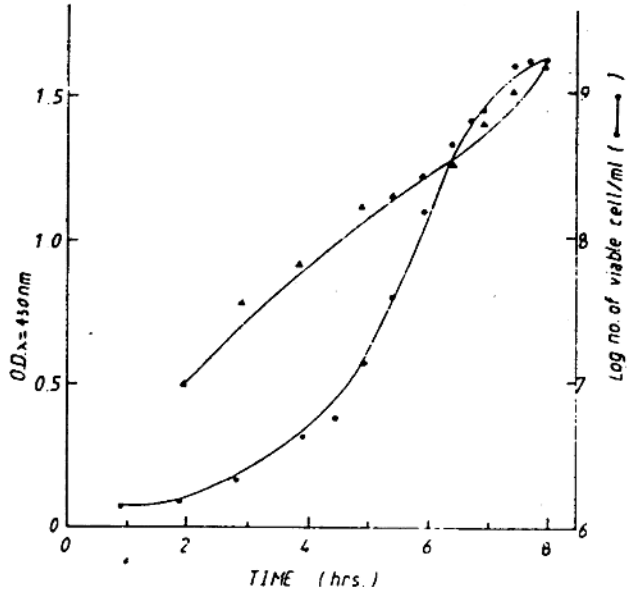

Fig. 1. Growth curve of *Edwardsiella tarda* at 28°C with aeration.
•—• Viable cells; ▲---▲ OD_{450} nm.

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	−	
810217-2I	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 $OD_{550nm}=1.0$, 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 1×10^4/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.

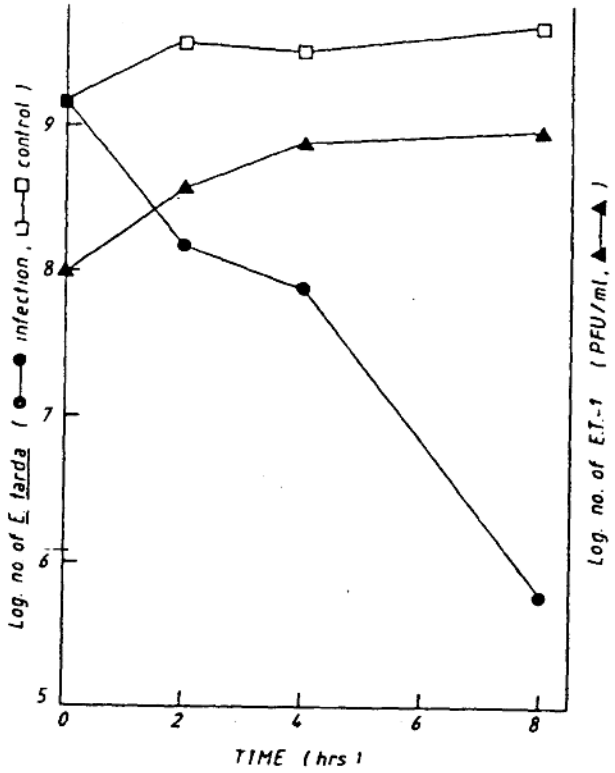

Fig. 2. Killing of *Edwardsiella tarda* and multiplication of bacteriophage ϕET-1.

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-