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# ISOLATION AND CHARACTERIZATION OF *EDWARDSIELLA ICTAURI*-SPECIFIC BACTERIOPHAGES

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## ABSTRACT

The present study aimed to isolate and characterize bacteriophages (phages) specific for *Edwardsiella ictaluri* to be used as a biological control of the infection in fish as a final goal. Twenty-six phages (PEi1 ~ PEi26), lytic to *E. ictaluri* were isolated from ayu *Plecoglossus altivelis* and its surrounding environment (water and mud) using enrichment and double agar layer methods. All phages formed clear plaques ranging in size from 0.3 to 8 mm in diameter. Transmission electron microscopy revealed that all the isolated bacteriophages belonged to the family *Myoviridae*. According to phage morphology, plaques size and DNA restriction patterns with *Eco*RI, the phages were classified into four groups (I ~ IV). *E. ictaluri* strains isolated from ayu (n=57) were sensitive to these phages with various degrees, resulting in twenty-five phage types of the bacterium. *E. ictaluri* strains isolated from catfish in the USA (type strain; JCM1680), Indonesia (n=4) or Vietnam (n=2) exhibited no or very limited susceptibility to the present phages. In addition, *E. tarda*, the most closely related species to *E. ictaluri*, as well as other fish pathogens were not susceptible to any of the phages examined.

***Key words:*** *E. ictaluri* specific-bacteriophage, TEM, phage typing, host range, restriction enzyme

## INTRODUCTION

*Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC), was first isolated from pond-reared channel catfish *Ictalurus punctatus* in 1976 in the USA (Hawke, 1979), characterized and classified by Hawke *et al.* (1981). Subsequently, *E. ictaluri* was reported from several natural outbreaks in non-ictalurid hosts including green knife fish *Eigemannia virscens* (Kent and Lyons, 1982), danio *Danio devario* (Waltman *et al.*, 1985), and tadpole madtom *Noturus gyrinus* in the United States (Klesius *et al.*, 2003), walking catfish *Clarias batrachus* in Thailand (Kasornchandra *et al.*, 1987), striped catfish *Panagassius hypophthalmus* in Vietnam (Crumlish *et al.*, 2002; Ferguson *et al.*, 2001) and in Indonesia (Yuasa *et al.*, 2003), rainbow trout *Oncorhynchus mykiss* in Turkey (Keskin *et al.*, 2004), yellow catfish *Pelteobagrus fulvidraco* in China (Ye *et al.*, 2009) and the bacterium was recently isolated from wild Australian catfish *Tandanus tropicanus* (Kelly *et al.*, 2018).

In Japan, *Edwardsiella ictaluri* was isolated for the first time in 2007 from natural outbreaks in wild Japanese ayu *Plecoglossus altivelis* populations and the disease was described as *E. ictaluri*-infection in ayu (Sakai *et al.*, 2008). Subsequently, phenotypical and genetic characterization of the isolated strains were carried out. All the tested isolates (n=4) were identical in morphological, physiological, biochemical and genetic characters (Nagai *et al.*, 2008). Moreover, the pathogen was isolated from apparently healthy wild ayu and forktail bullhead *Pseudobagrus nudiceps* from Gonokawa River in Hiroshima Prefecture, Japan in two successive years; 2008 and 2009, with isolation rate of 30.5% and 23.5% respectively (Hassan *et al.*, 2012). All *E. ictaluri* isolates (n=128) from ayu and a strain from forktail bullhead, collected from different localities in Japan, were identical physiologically, biochemically; except for $H_2S$ production; and genetically using the partial nucleotide sequences of a Type 1 fimbrial gene cluster (*etfA*, *etfB*, *etfC*, *etfD*), and random amplified polymorphic DNA (RAPD) analysis (Hassan *et al.*, 2012). Also, these isolates were serologically the same (Hassan *et al.*, 2010). Thus, another method for differentiation of these strains was still needed. Therefore, the current work was directed to isolate and characterize *E. ictaluri*-specific phages for further characterization of the present *E. ictaluri* strains isolated from ayu. Also, detection of specific phages may help in control of infection caused by this pathogen.

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## MATERIALS AND METHODS

**Fish Samples:**
A total number of 295 apparently healthy wild ayu (average standard length ± standard deviation [SD] of 178.0±19.0 mm and average body weight ± SD of 81.8±26.0 g) were caught by gill net from the Gonokawa River, Miyoshi City-Hiroshima Prefecture, Japan. The fish were collected monthly (average 25 fish per month). Fish were transported to the Laboratory of Fish Pathology, Hiroshima University, on ice, and phage isolation from kidney was carried out.

**Environmental samples:**
Water and mud samples (200 mL; n=12) were collected in sterile bottles from the same place of the fish sampling (Gonokawa River, Myioshi city-Hiroshima Prefecture). Also, pond water samples (200 mL) were collected from cultured ayu farms (n=11) in Tokushima, Shiga and Wakayama prefectures and subject to bacteriophage isolation.

**Bacterial strains and media:**
Strains of *E. ictaluri* used in this study are listed in Table 1. They include strains isolated from wild ayu at different localities in Japan, the type strain JCM1680 (ATCC33202) of *E. ictaluri* isolated from channel catfish, and six *E. ictaluri* strains isolated from striped catfish in Indonesia, and Vietnam (Hassan *et al.*, 2012). All strains were further confirmed as *E. ictaluri* using using polymerase chain reaction (PCR) with an *E. ictaluri*-specific primer set, EDi (EDi-F: 5´-CAGATGAGCGGATTTCACAG- 3´; EDi-R: 5´-CGCGCAATTAACATAGAGCC-3´), that targets the upstream region of the fimbrial gene cluster (470 bp) as was described by Sakai *et al.* (2009). Also, four strains of the closely related fish pathogen, *E. tarda* including the type strain (JCM1656= ATCC15947), and some other fish pathogens were used (Table 1). Heart infusion broth (HIB; Nissui, Japan), soft agar (Trypto-soy broth, TSB; Eiken, Japan supplemented with 0.35% agar) and Trypto-soya agar (TSA; Nissui) were used for bacterial culture and bacteriophage plaque forming unit (PFU) assay (Carlson, 2005). All strains were kept at −80°C in HIB containing 25% glycerol until being used. Prior to experiments, the strains were inoculated on TSA directly from −80°C and incubated at 30º C for 48 hours.

**Isolation of *E. ictaluri* phage and PFU assay:**
An enrichment method described previously (Carlson, 2005) was employed to isolate *E. ictaluri* phages from the natural environment (water and mud) as well as from the kidney of apparently healthy wild ayu. In brief, pooled kidney samples from wild ayu were inoculated in HIB supplemented with *E. ictaluri* as host cells. Unless otherwise mentioned, five strains of *E. ictaluri*; D4, AH0801, AH0816, PH0744 isolated from wild ayu in addition to Oth.29 isolated from forktail bullhead (Hassan *et al.*, 2012), were used as host bacteria. Each environmental sample (200 ml) was mixed with the same volume of double strength HIB provided with the host cells. After being incubated at 25ºC overnight in a static condition, the culture was centrifuged at 5,000 rpm for 30 min at 4 °C., and the supernatant was filtered using 0.45 µm cellulose filter (Advantec®, Japan). The presence of lytic phages in the filtrate was detected by the spot method with the double agar layer (Carlson, 2005). Briefly, 400 µl of *E. ictaluri* cells in the exponential growth phase were mixed with 3 ml of 50°C top soft agar (TSB with 0.35% agar), and the mixture was poured onto a TSA plate. After solidification (around 30 min), 20 µl of the filtrate (spot) was inoculated onto the double agar layer plate and the plate was incubated at 25°C overnight. The sample was scored positive for the presence of lytic phage if a clear zone or plaque was detected in the bacterial lawn of the plate.

**Phage cloning:**
A single plaque was picked up with a sterile loop and inoculated into HIB supplemented with one strain of *E. ictaluri*. Each phage was isolated as a single clone by three cycles of plaque purification and re-infection. For determination of phage concentration, 10-fold serial phage dilutions in phosphate buffered saline (PBS, 0.01 M, pH 7.4) were used and inoculated onto double agar layer (Carlson, 2005). All assays were performed in triplicates.

**Electron microscopy of phage:**
Following the method described by Kawato *et al.* (2015), fifteen microliters of a phage suspension were spotted on top of a formvar-carbon-coated copper grid, and the phage was allowed to adsorb for 10 seconds. Excess amount was removed carefully by touching the side of the grid with filter paper. The sample was washed three times with sterile filtered distilled water and stained by the addition of 8 µL of 2% uranyl acetate (pH 4). Excess stain was removed, and the grid was allowed to air dry for 10 min. The grids were observed using transmission electron microscope (JEOL, JEM-1200EX, Japan) at 80 KV.

**Determination of the phage host range:**
Strains of *E. ictaluri* (n=57) isolated from ayu (Hassan *et al.*, 2012), Japan, were used in this study (Table 1). Other *E. ictaluri* strains from Indonesia (n=4), Vietnam (n=2), one strain from the USA as well as some other fish pathogenic bacteria including *E. tarda* (n=4), *Vibrio parahemolyticus, Pseudomonas plecoglossicida*, *Streptococcus iniae,* and *Lactococcus garvieae* were also used for comparison. The host ranges of the isolated phages were determined by the spot method (Carlson, 2005) where 20 µl containing $5\times10^5$ PFU of each phage

suspension was spotted on top agar layer of TSA plate that is freshly prepared with 3 ml of top agar inoculated with 400 µl of the strain to be tested. The host range was determined with three separate plates for each phage-host combination, and all bacterial strains were tested against all the phage isolates.

**Analysis of the phage nucleic acids:**

**Extraction and purification of phage nucleic acid:**

Extraction and purification of phage nucleic acid was carried out as described previously (Carlson, 2005) with slight modification. Briefly, phage was propagated with its host strain in 300 ml of HIB medium and incubated at 25°C overnight. After removal of the bacterial debris by mild centrifugation at 5,000 rpm at 4 °C for 30 min and passage through 0.45 µm filter, the filtrate was supplemented with polyethylene glycol 6000 (Nacalai Tesque, Japan) and NaCl at final concentrations of 10% and 0.5 M, respectively, and incubated at 4 °C overnight. Phage was precipitated by centrifugation (15,000 rpm, 4°C, 15 min) and the precipitate was suspended in 0.5 ml diluent [0.01 M Tris-HCl (pH 7.4), 0.15 M NaCl, 0.03% (w/v) gelatin]. Finally, phage was sequentially purified by cesium chloride (CsCl) step gradient ultracentrifugation (45,000 rpm, 4°C, 18 h) using the S80AT3 rotor and a GX series Himac CS 100GX microultracentrifuge (Hitachi Ltd., Tokyo, Japan). After collection of phage band, the purified phage was dialyzed with PBS (0.01 M, pH 7.4) three times at 1-hour interval. To the dialyzed phage, proteinase K was added to a final concentration of 50 µg/ml and sodium dodecyl sulfate (SDS) to a final concentration of 0.5% and incubated at 56°C for 1 hour. The protein was removed by two phenol saturated TE buffer (l0 mM Tris-HCl, pH 8.0, 1 mM EDTA), chloroform-isoamyl alcohol (25:24:1) extractions. The nucleic acid was precipitated by the addition of a 0.1 volume of 3 M sodium acetate and a 2.5 volume of absolute cold ethanol and the mixture was incubated at -20°C overnight, followed by centrifugation at 14,000 rpm (10 min, 4°C). The DNA pellet was washed twice with 70% ethanol, dried and finally dissolved in 50 to 100 µL of TE buffer (pH 8.0).

**Restriction enzyme digestion of the phage nucleic acids:**

The purified nucleic acid was tested for sensitivities to RNase A (20 µg/ml), DNase I (20 µg/ml), and the restriction enzyme *Eco*RI (20 µg/mL) by incubation at 37ºC for 30 min. Samples were analyzed along with undigested phage DNA. The resulting product was electrophoresed through 1% agarose gel at 50 V and visualized by ethidium bromide.

## RESULTS

**Phage isolation:**

A total of 26 *E. ictaluri*-lytic phages (Table 2) were isolated from wild ayu (kidney) and the surrounding environment (mud and water) by the enrichment method. The isolated phages were designated as PEi1~PEi26. They formed clear plaques on double agar layer ranged in size from 0.3 to 8 mm in diameter (Fig. 1). *E. ictaluri* phages were found in all samples examined from wild ayu and their natural environment but not from cultured ayu (n=11 farms) in Wakayama, Shiga and Tokushima prefectures.

**Phage morphology:**

Transmission electron microscopy of negatively-stained phage isolates revealed that all of them belonged to family *Myoviridae* that is characterized by head and contractile tail with spikes (Table 3, Fig. 2). According to the number of phage isolates within each group, phages were classified into four groups (group I~IV) where group I included the majority of the isolated phage (n= 19) that produced clear plaques on double agar layer, ranged from 1.5-2.5 mm in diameter and characterized by isometric head and relatively short tail (< 60 nm); group II comprised five phage isolates that produced hazy big plaques (5-8 mm in diameter) and characterized by isometric head and relatively larger tail (> 80nm); group III consisted of two phage isolates that produced round clear plaques (0.5-1 mm in diameter) and had big elongated head and long tail (> 120nm), and group IV that had only one phage and form very small plaques (0.3 - 0.4 mm in diameter) and characterized by elongated head and long tail (>120 nm; Fig. 1 and 2).

**Host range and phage typing of the bacterium:**

The host ranges of the isolated phages, tested against different *E. ictaluri* strains (Table 1) isolated from different fish species, are shown in Fig. 3. The phages were able to lyse all *E. ictaluri* strains isolated from ayu (Japan), but not those isolated from other countries (USA, Indonesia, and Vietnam) and this resulted in twenty-five phage types of the bacteria with two major phage types; phage type one and two that were represented respectively by 13 and 12 strains of *E. ictaluri* isolated from ayu (Table 4). None of the tested *E. tarda* strains as well as the other fish pathogenic bacteria was lysed by any of these phages.

**Analysis of the phage nucleic acids:**

The nucleic acids of the isolated phages were digested by DNase I but not by RNase A and cleaved by *Eco*RI (except EPi20 and EPi26) resulting in various restriction patterns among different phage groups and same restriction pattern among individuals of the same group (Fig.4).

**Table 1.** Sources of bacterial strains used in this study

| Bacterial strain | Source | | |
|---|---|---|---|
| | Host fish | Locality | Year |
| *Edwardsiella ictaluri* | | | |
| JCM1680 [a] | Channel catfish [b] | Georgia, USA | 1976 |
| JF0207 | Striped catfish [c] | Indonesia | 2002 |
| JF0381 | Striped catfish | Indonesia | 2003 |
| JF0384 | Striped catfish | Indonesia | 2003 |
| JF0378 | Striped catfish | Indonesia | 2003 |
| DTHN01 | Striped catfish | Vietnam | 2009 |
| DTHN04 | Striped catfish | Vietnam | 2009 |
| FPC1091 | Diseased Ayu [d] | Yamaguchi Pref., Japan | 2007 |
| FPC1092 | Diseased Ayu | Tokyo, Japan | 2007 |
| FPC1093 | Diseased Ayu | Tokyo, Japan | 2007 |
| PH0744 (FPC1094) | Diseased Ayu | Hiroshima Pref., Japan | 2007 |
| FPC1095 | Ayu | Shimane Pref., Japan | 2008 |
| FPC1096 | Ayu | Shimane Pref., Japan | 2008 |
| FPC1097 | Ayu | Shimane, Pref. Japan | 2008 |
| FPC1098 | Ayu | Gifu Pref., Japan | 2008 |
| FPC1099 | Ayu | Tottori Pref., Japan | 2008 |
| FPC1100 | Ayu | Tottori Pref., Japan | 2008 |
| FPC1101 | Ayu | Tokyo, Japan | 2008 |
| FPC1102 | Ayu | Tokyo, Japan | 2008 |
| FPC1103 | Ayu | Okayama Pref., Japan | 2008 |
| D4 | Dead Ayu | Hiroshima Pref., Japan | 2008 |
| AH0801-AH0890 (n=90) | Healthy Ayu | Hiroshima Pref., Japan | 2008 |
| AH0901-AH0954 (n=12) | Healthy Ayu | Hiroshima Pref., Japan | 2009 |
| Oth.29 | Forktail bullhead[e] | Hiroshima Pref., Japan | 2008 |
| 0801 | Ayu | Tokushima Pref., Japan | 2008 |
| 0803 | Ayu | Tokushima Pref., Japan | 2008 |
| KY-7 | Ayu | Kyoto Pref., Japan | 2009 |
| WY-2 | Ayu | Wakayama Pref., Japan | 2009 |
| TY-1 | Ayu | Toyama Pref., Japan | 2009 |
| TG-7 | Ayu | Tochigi Pref.., Japan. | 2009 |
| KC-4 | Ayu | Kochi Pref., Japan | 2009 |
| GF-2 | Ayu | Gifu Pref., Japan | 2009 |
| *E. tarda* | | | |
| FK1051 | Japanese flounder | Hiroshima Pref., Japan | 2003 |
| NUF251 | Japanese flounder | Nagasaki Pref., Japan | 1986 |
| SU-100 | Japanese eel | Shizuoka Pref., Japan | 1980 |
| JCM1656 [f] | Type strain | | 1982 |
| *Vibrio parahemolyticus* | Ayu | Japan | 1987 |
| *Pseudomonas plecoglossicida* | Ayu | Hiroshima Pref., Japan | 1995 |
| *Streptococcus iniae* | Japanese flounder | Japan | 1983 |
| *Lactococcus garvieae* | Yellowtail | Ehime Pref., Japan | 2002 |

[a]JCM, Japan Collection of Microorganisms (=ATCC33202), [b]*Ictalurus punctatus* [c],*Pangasius hypophthalmus* [d], *Plecoglossus altivelis*, [e] *Pseudobagrus nudiceps*, [f] =ATCC15947.

**Table 2.** *Edwardsiella ictaluri* phages isolated from wild ayu (kidney) and its surrounding environment

| Isolation from | Phage No. | *E. ictaluri* (host cell) | Plaque size (mm) | Bacterial No. lysed by phage (out of 57) |
|---|---|---|---|---|
| **Fish** | PEi3 | AH0801 | 1.5-2.5 | 46 |
| | PEi6 | Oth29 | 1.5-2.5 | 51 |
| | PEi13 | AH0816 | 1.5-2.5 | 51 |
| | PEi23 | D4 | 1.5-2.5 | 50 |
| **Water** | PEi2 | Oth29 | 1.5-2.5 | 51 |
| | PEi4 | AH0801 | 1.5-2.5 | 51 |
| | PEi7 | Oth29 | 1.5-2.5 | 51 |
| | PEi8 | Oth29 | 1.5-2.5 | 51 |
| | PEi10 | AH0816 | 1.5-2.5 | 44 |
| | PEi12 | D4 | 1.5-2.5 | 49 |
| | PEi14 | D4 | 1.5-2.5 | 51 |
| | PEi15 | PH0744 | 1.5-2.5 | 51 |
| | PEi17 | Oth29 | 0.3-0.4 | 51 |
| | PEi18 | Oth29 | 5-8 | 18 |
| | PEi21 | AH0816 | 5-8 | 24 |
| | PEi22 | AH0816 | 1.5-2.5 | 52 |
| | PEi25 | AH0816 | 5-8 | 43 |
| **Mud** | PEi1 | Oth29 | 1.5-2.5 | 51 |
| | PEi5 | Oth29 | 1.5-2.5 | 51 |
| | PEi9 | AH0816 | 1.5-2.5 | 51 |
| | PEi11 | PH0744 | 1.5-2.5 | 49 |
| | PEi16 | Oth29 | 1.5-2.5 | 51 |
| | PEi19 | Oth29 | 5-8 | 42 |
| | PEi20 | Oth29 | 0.5-1 | 49 |
| | PEi24 | Oth29 | 1.5-2.5 | 50 |
| | PEi26 | AH0816 | 0.5-1 | 49 |

**Table 3.** Morphological characteristics of the isolated *Edwardsiella ictaluri* phages

| Group | Head diameter (nm) | Head length (nm) | Tail diameter (nm) | Tail length (nm) | Family |
|---|---|---|---|---|---|
| I | 55.2±12.2 | 52.5±8.1 | 19.5±2.0 | 59.6±2.8 | *Myoviridae* |
| II | 69.4±3.9 | 66.6±0.0 | 22.15±7.8 | 81.95±9.8 | *Myoviridae* |
| III | 87.4±5.0 | 100.8±9.3 | 28.6±3.8 | 129.7±8.2 | *Myoviridae* |
| IV | 80.6±4.8 | 109.8±4.7 | 26.4±2.4 | 127.7±9.6 | *Myoviridae* |

**Table 4.** Phage typing scheme of *Edwardsiella ictaluri*

| Phage group No. | PEi1 | PEi2 | PEi3 | PEi4 | PEi5 | PEi6 | PEi7 | PEi8 | PEi9 | PEi10 | PEi11 | PEi12 | PEi13 | PEi14 | PEi15 | PEi16 | PEi17 | PEi18 | PEi19 | PEi20 | PEi21 | PEi22 | PEi23 | PEi24 | PEi25 | PEi26 | Bacterial strains No. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | □ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | 13 |
| 2 | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | □ | ■ | ■ | □ | ■ | ■ | ■ | ■ | ■ | 12 |
| 3 | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | □ | ■ | ■ | □ | ■ | ■ | ■ | □ | ■ | 4 |
| 4 | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | □ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | 3 |
| 5 | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | □ | ■ | ■ | □ | ■ | ■ | ■ | ■ | □ | 2 |
| 6 | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | □ | ■ | ■ | □ | ■ | ■ | ■ | □ | □ | 2 |
| 7 | ■ | ■ | □ | ■ | ■ | ■ | ■ | ■ | ■ | □ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | □ | □ | ■ | □ | ■ | ■ | ■ | ■ | ■ | 2 |
| 8 | ■ | ■ | □ | □ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | 1 |
| 9 | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | □ | ■ | □ | ■ | 1 |
| 10 | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | □ | ■ | □ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | 1 |
| 11 | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | □ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | □ | □ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | 1 |
| 12 | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | □ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | □ | □ | 1 |
| 13 | ■ | ■ | □ | ■ | ■ | ■ | ■ | ■ | ■ | □ | ■ | ■ | ■ | ■ | ■ | ■ | □ | □ | □ | ■ | □ | ■ | ■ | □ | ■ | ■ | 1 |
| 14 | □ | □ | ■ | □ | □ | □ | □ | □ | □ | ■ | ■ | ■ | □ | □ | □ | □ | ■ | □ | □ | ■ | □ | □ | □ | ■ | □ | □ | 1 |
| 15 | □ | □ | ■ | ■ | □ | □ | □ | □ | □ | ■ | ■ | ■ | ■ | □ | ■ | ■ | ■ | □ | ■ | ■ | ■ | ■ | □ | ■ | ■ | ■ | 1 |
| 16 | □ | □ | □ | □ | □ | □ | □ | □ | □ | □ | □ | ■ | □ | □ | □ | □ | ■ | □ | ■ | ■ | □ | □ | □ | ■ | ■ | □ | 1 |
| 17 | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | □ | □ | □ | ■ | ■ | ■ | ■ | ■ | ■ | 1 |
| 18 | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | □ | □ | □ | ■ | ■ | ■ | ■ | ■ | □ | □ | □ | □ | ■ | ■ | ■ | ■ | ■ | 1 |
| 19 | ■ | ■ | □ | ■ | ■ | ■ | ■ | ■ | ■ | □ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | □ | ■ | ■ | □ | ■ | ■ | ■ | □ | ■ | 1 |
| 20 | □ | □ | □ | □ | □ | □ | □ | □ | □ | □ | □ | □ | □ | □ | □ | □ | ■ | □ | ■ | ■ | ■ | □ | □ | ■ | □ | ■ | 1 |
| 21 | □ | □ | □ | □ | □ | ■ | ■ | □ | □ | □ | □ | □ | □ | □ | ■ | ■ | □ | □ | ■ | ■ | □ | □ | □ | □ | ■ | □ | 1 |
| 22 | ■ | ■ | □ | □ | ■ | ■ | ■ | ■ | ■ | □ | □ | □ | □ | ■ | □ | □ | □ | □ | □ | □ | □ | □ | ■ | □ | □ | □ | 1 |
| 23 | ■ | ■ | ■ | □ | ■ | ■ | ■ | ■ | □ | ■ | ■ | ■ | □ | ■ | □ | □ | ■ | □ | ■ | □ | □ | ■ | □ | ■ | □ | □ | 1 |
| 24 | ■ | ■ | ■ | □ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | □ | ■ | □ | □ | ■ | □ | ■ | □ | □ | □ | ■ | ■ | □ | □ | 1 |
| 25 | ■ | ■ | ■ | ■ | ■ | ■ | ■ | □ | □ | ■ | ■ | ■ | ■ | □ | ■ | ■ | ■ | □ | ■ | ■ | □ | ■ | ■ | ■ | ■ | ■ | 1 |

■ Sensitive □ Resistant

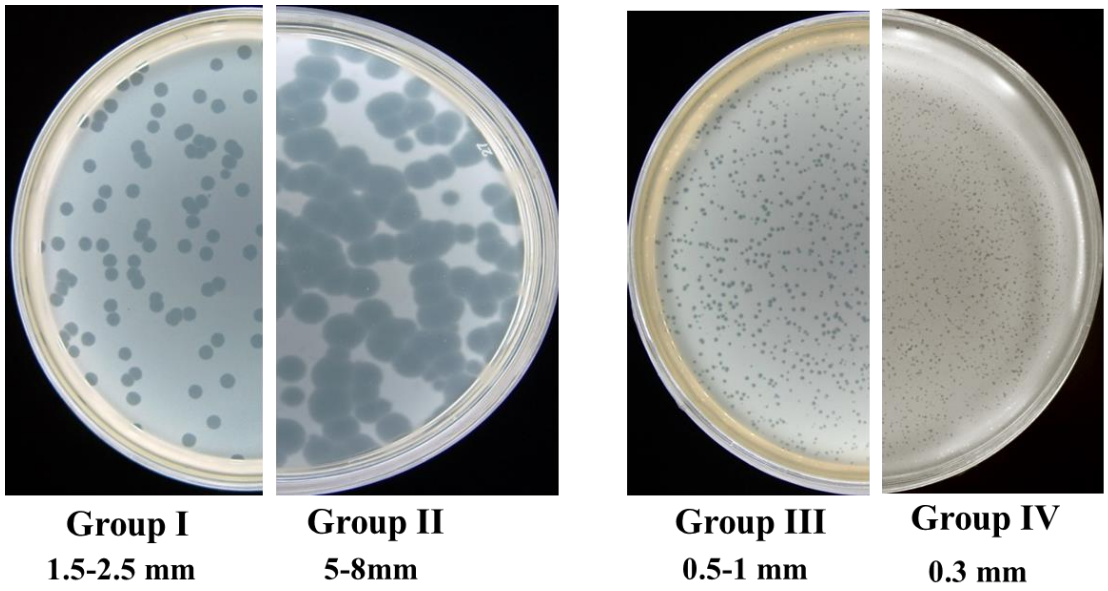


**Fig. 1:** Plaques of representative *Edwardsiella ictaluri* bacteriophages on a double agar layer. Phages were classified into four groups based on their abundance and plaque sizes.

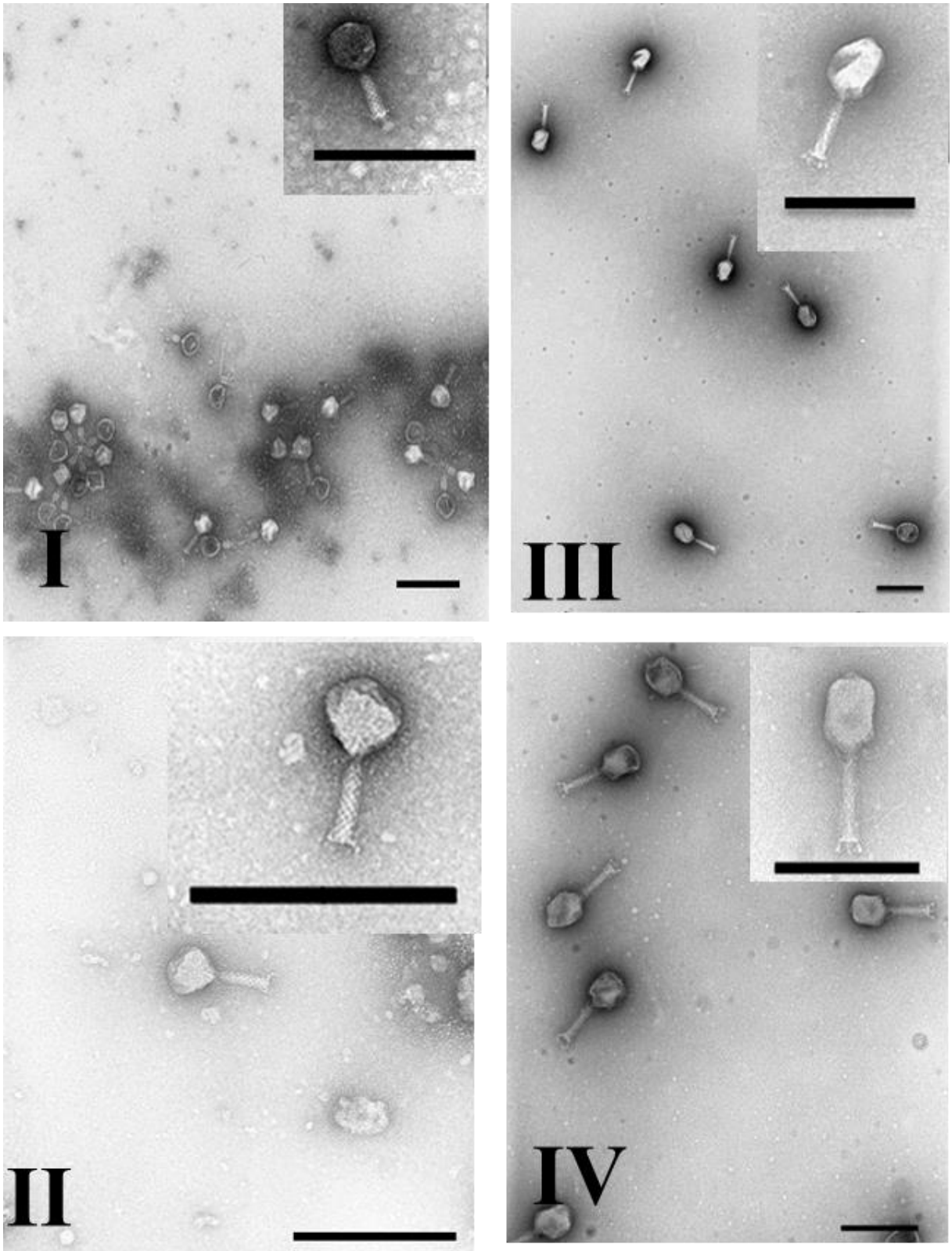


**Fig. 2:** Transmission electron microscopy (TEM) picture of representative *Edwardsiella ictaluri* phages (A) PEi1 (Group I); (B) PEi19 (Group II); (C) PEi17 (Group III); (D) PEi20 (Group IV). Scale bar=200 nm

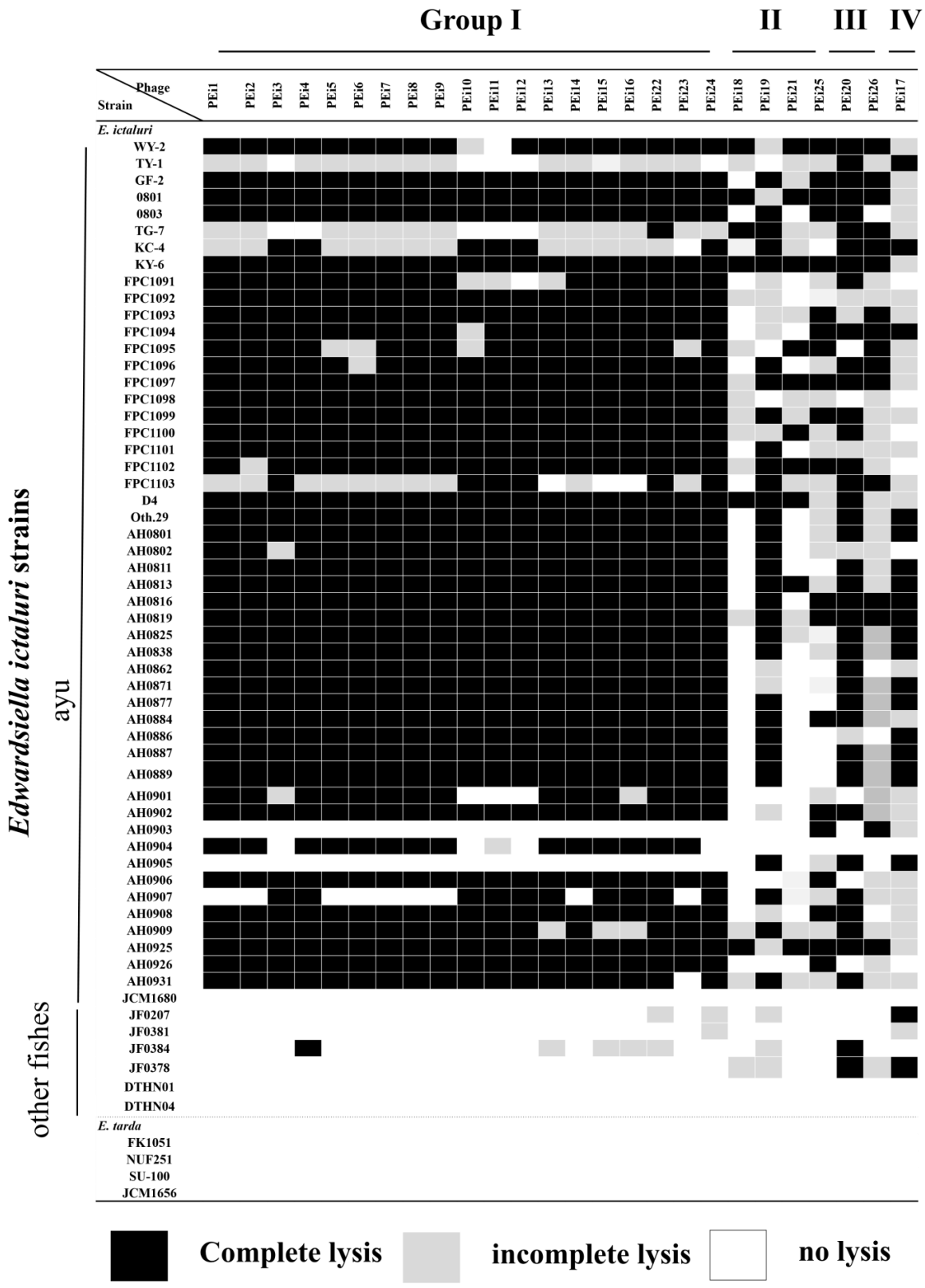


**Fig. 3:** Host range of the isolated phages against representative *Edwardsiella ictaluri* strains.■: complete lysis, ▒: incomplete lysis, □: no-lysis.

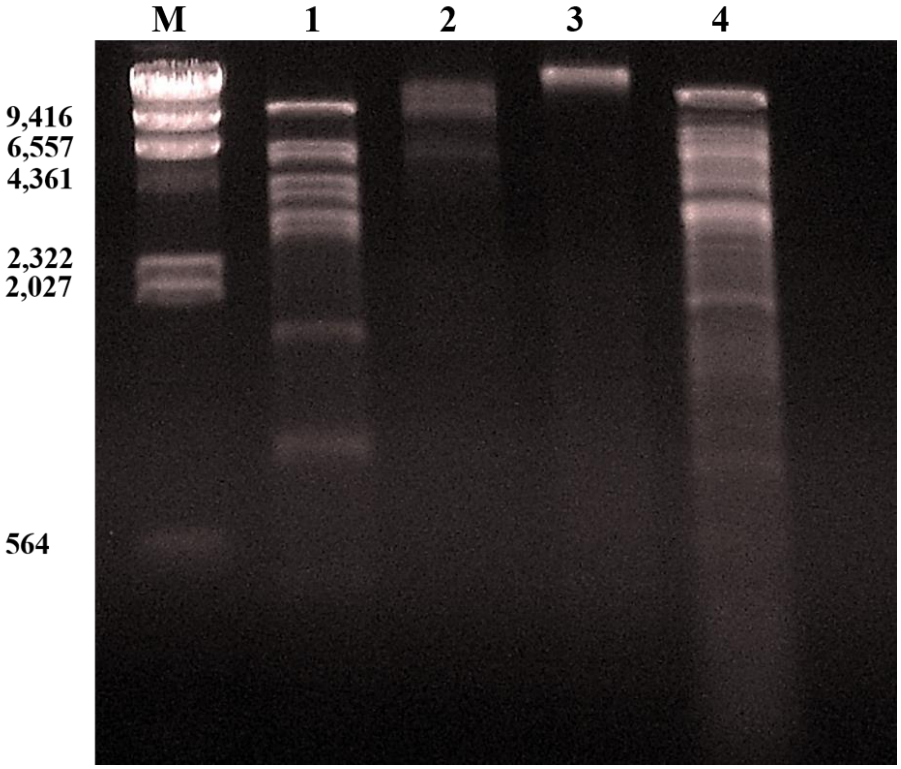


**Fig. 4:** Agarose gel electrophoresis of restrictions digest analysis of representative *Edwardsiella ictaluri* bacteriophages after treatment with *Eco*RI. M: Marker (λ/*Hin*d III digests), 1: PEi1 (Group I) phage, 2: PEi19 (Group II) phage, 3: PEi20 (Group III) phage and 4: PEi17 (Group IV) phage.

## DISCUSSION

The current study describes the isolation of 26 bacteriophages specific to *E. ictaluri* isolated from wild ayu, showing no clinical signs, and its environment (water, and mud), using enrichment method. These phages may contribute to lessening the incidence of *E. ictaluri* outbreaks. Since *E. ictaluri* is also reported to survive in water and pond bottom sediments for a relatively long time (Plumb and Quinlan, 1986), there is a reason to suspect that *E. ictaluri*- specific phages may persist in the environment. In our previous work, a semi-quantitative method was conducted, on a monthly basis, for the estimation of *E. ictaluri* phage concentration using 1, 10, 50, 100, 200, and 400 ml river water. We could detect *E. ictaluri* phages even in 1 ml river water (Hassan *et al.*, 2012). Moreover, *E. ictaluri* phages could be isolated throughout a one-year study period indicating the coexistence of *E. ictaluri* and its phage(s) in the river water (Hassan *et al.*, 2012). This result comes with the idea that bacteriophages are ubiquitous in the environments inhabited by their respective bacterial host(s) (Kutter and Sulakvelidze, 2005). In addition,Walakira and coworkers described the isolation and the characterization of two unique *E. ictaluri*-specific phagesof family *Siphoviridae*; ΦeiAU (eiAU) and ΦeiDWF (eiDWF) from aquaculture ponds with a history of ESC (Walakira *et al.*, 2008). Sebsequently, theses phages were sequenced (Carrias *et al.*, 2011).

In the present study, the enrichment method, used for the phage isolation from ayu and its environment, was proved to be effective. Also, Walakira *et al.* (2008) isolated *E. ictaluri*-specific phages by concentrating viruses from pond water samples by ultrafiltration and enriching for *E. ictaluri*-specific bacteriophages via enrichment in log-phase bacterial broth cultures.

The phages described herein displayed plaques of different sizes (0.3-8 mm in diameter) on the double agar layer. However, the transmission electron microscopy revealed similar morphotypes, classified as members of *Myoviridae* by the phage taxonomy (Ackermann, 2005). All the isolated phages belonged to family *Myoviridae* that had icosahedral or elongated head and a contractile tail structure that is more or less rigid, long and relatively thick, and consists of a central core built of stacked rings of six subunits and surrounded by a helical contractile sheath, which is separated from the head by a neck. During contraction, sheath subunits slide over each other and the sheath becomes shorter and thicker, which brings the tail core in contact with the bacterial plasma membrane. Myoviruses tend to be larger than other groups and include some of the

largest and most highly evolved tailed phages (Martha and Andrew, 2009).

All phage isolates were DNA phages as they were digested by DNase I but not by RNase A. The phage nucleic acids were larger than 20,000 bp and reached up to 43,378 bp in PEi21 (Yasuike *et al.*, 2014). Similar data was reported by Carrias and coauthors who reported that the whole genomes of *E. ictaluri* phages; eiAU, eiDWF, and eiMSLS were 42.80 kbp, 42.12 kbp, and 42.69 kbp, respectively (Carrias *et al.*, 2011).

The restriction digests using *Eco*RI showed that the cleaved nucleic acids of the phages exhibited various restriction patterns among the different phage groups and same pattern among individuals of the same group, however, group III phages that is represented by PEi20 and PEi26 was not digested by endonucleases, and this may be due to methylations or glucosylations on the phage DNA (Nelson and McClelland, 1991). Based on these results, the isolated phages were classified into four groups; the first group which was the main group and contained 19 phage isolates. It was widespread as it was isolated from ayu kidneys, river water, and mud forming clear round plaques having sharp edges with an average diameter of 1.5-2.5 mm and the host bacteria were *E. ictaluri* isolated from wild ayu. The second group represented by five phage isolates, produced big hazy plaques (around 5-8 mm in diameter) and their host was a resistant *E. ictaluri* isolate evolved both *in vivo* and *in vitro*. The third group included two phage isolates that were isolated from river mud only. Phages of this group displayed small plaques (0.5 - 1 mm in diameter) and the host bacteria is a resistant *E. ictaluri* isolate evolved *in vivo*. The fourth group consisted of only one phage isolate that was isolated from river water and its host cell was a resistant *E. ictaluri*, retrieved *in vitro*, and form very small round plaques with average 0.3-0.4 mm in diameter. Some of the isolated phages produced clear plaques suggesting that they may be lytic and virulent to *E. ictaluri.* The lytic characters of a representative phage was discussed in a previous work (Yasuike *et al.*, 2015) who reported the absence of lysogeny- related genes such as integrase, repressor CI, regulatory protein CII and anti-repressors in *E. ictaluri* phage, PEi21.

Phage typing is a well-known sensitive tool for establishing relationships among intra-species of bacteria (Pfaller, 1991). Interestingly, all the tested *E. ictaluri* strains (n=57), isolated from apparently healthy wild ayu at different localities in Japan (Hassan *et al.*, 2012), were sensitive to the isolated phages but with different degrees resulting in twenty-five phage types (1 ~ 25), with major phage types; phage type one represented by 13 *E. ictaluri* strains, and phage type two represented by12 *E. ictaluri*-sensitive strains. The variation in susceptibility among host strains may be largely due to differences in host receptor sites, modification or loss of receptor molecules, or other host resistant mechanisms such as abortive infection (Levin and Bull, 2004). It is noteworthy to mention that *E. ictaluri* strains isolated from catfish in the USA (one isolate), Indonesia (n=4) or Vietnam (n=2) exhibited very limited susceptibility to the present phages. In addition, *E. tarda*, the most closely related species to *E. ictaluri* (Waltman *et al.*, 1986), was not susceptible to any of the phages examined in the current study., indicating that all the isolated *E. ictaluri*-specific phages may be a useful diagnostic tool for *E. ictaluri* strains isolated from nearby geographic but not distant areas.

Screening the lytic activity of these phages, revealed that PEi22 was one of the most potent phages lysing 52 out of the 57 strains examined, in which 42 strains were lysed efficiently indicated by clear plaques. Also, PEi20 could lyse 49 out of the 57 strains. At the other end of the range, PEi18 infected only 18 strains eliciting clear plaques with only six strains. Compared to chemotherapeutants that have a broad-spectrum activity on different species or strains (Nelson, 2004), the use of a strain-specific phage may be not effective to control the pathogen in question, yet a 'cocktail' of *E. ictaluri* specific phages may have better efficiency as a biological control strategy (O'Flynn *et al.*, 2004). A combination of phages with different lytic properties may thus provide the best starting point for further exploration of the potential of phage therapy for controlling pathogenic bacteria. In the present study, we isolated a suite of lytic *E. ictaluri* phages that were able to infect and lyse a wide range of *E. ictaluri* strains. The most potent phages were PEi4, PEi19, PEi20, PEi22, and PEi26 which together lyse almost all *E. ictaluri* strains examined. Therefore, the mixture of these powerful phages, having a prominent lytic effect, may help for control of *E. ictaluri* infection in cultured fish. In a prelimnary study to explore the role of the isolate phages to control *E. ictaluri* infection in ayu, when ayu was first injected with virulent *E. ictaluri* strain and one-hour later injected with a mixture of these phages, a delayed mortality, in comparison with the control was noticed. On the other hand, high protection was observed in fish that was first injected with phages and then injected with the pathogen. These results endorsed the use of these phages in phage therapy of *E. ictaluri* infection of ayu (Mahmoud and Nakai, 2012).

In conclusion, we could isolate 26 *E. ictaluri*-lytic phages from wild ayu and its surrounding environment, and identify these phages both morphologyically and by the restriction enzyme that is a prerequiste for evaluating the phage potential as a biological control of a pathogenic host. Also, these phages served in the differentiation of various *E.*

*ictaluri* strains isolated from different localities all over Japan. Furthermore, their promising strong lytic property both *in vitro* and the *in vivo* preliminary challenge provide a foundation for future exploration of their potential in treatment of *E. ictaluri* infection in fish farms to overcome the disadvantages of using antibiotics including their residues, side effects or developing of antibiotic-resistant strain(s).

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## عزل وتوصيف فيروسات آكلة بكتيريا الإدواردسيللا إكتالورى

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هدفت الدراسة الحالية إلى عزل وتوصيف فيروسات آكلة بكتيريا الإدواردسيللا إكتالورى بغرض استخدامها مستقبلياً فى التحكم البيولوجى فى عدوى ميكروب الإدواردسيللا إكتالورى فى الأسماك. تم عزل ٢٦ فيروس من أسماك الأيو والبيئة المحيطة بها (الماء و الطين). إستطاعت الفيروسات القضاء على البكتيريا وأحدثت مناطق خالية من البكتيريا على بيئة الزرع تراوحت فى أقطارها من 0.3 إلى ٨ ملليمتر. وباستخدام الميكرسكوب الإلكترونى، أظهرت النتائج أن كل الفيروسات تنتمى إلى عائلة الميوفيردي. وطبقاً لشكل الفيروس ونمط الجينوم باستحدام الانزيم *Eco*R1، تم تقسيم الفيروسات إلى ٤ مجموعات. أظهرت البكتيريا المعزولة من أسماك الأيو (٥٧ عترة) حساسية لهذه الفيروسات بدرجات مختلفة ونتج عن ذلك ٢٥ نمط من البكتيريا. فى حين أن البكتيريا المعزولة من أسماك أخرى فى الولايات المتحدة الأمريكية وإندونيسيا وفيتنام أظهرت عدم حساسية أو حساسية محدودة تجاه الفيروسات المستخدمة. هذا بالإضافة إلى أن ميكروب الإدواردسيللا تاردا- قريب الصلة بميكروب الإدواردسيللا إكتالورى- وكذلك ميكروبات أخرى لم يكن لها حساسية لأى من الفيروسات قيد البحث.