河川アユにおけるEdwardsiella ictaluri不顕性感染

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Subclinical *Edwardsiella ictaluri* Infection of Wild Ayu *Plecoglossus altivelis*

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**ABSTRACT**—*Edwardsiella ictaluri* infection is a newly emerging disease in ayu *Plecoglossus altivelis* in Japanese rivers, and has been continuously observed since the first outbreak in 2007. The present field study was performed in a river in Hiroshima Prefecture, Japan, where mortality due to *E. ictaluri* infection was recorded in 2007. Investigations over the course of 3 years (2008–2010) revealed the existence of constant subclinical *E. ictaluri* infection in apparently healthy ayu in the river, with higher rates (average 45.4%) in September and October, when water temperature drops and fish mature sexually. Among 11 other wild fish species examined at the same time, only one fork-tail bullhead *Pelleobagrus nudiceps* was positive for *E. ictaluri*. The bacterium was not isolated from groups of juvenile ayu just before the release into the river for stock enhancement of this species. On the other hand, the ubiquitous presence of *E. ictaluri*-specific phages in the river water and sediments suggested the existence of *E. ictaluri* in the river; that represents an environmental reservoir of the pathogen and may serve as a potential infection source for ayu. The current subclinical status of wild ayu infected with *E. ictaluri* might turn to overt infection and thus cause mortality under unidentified stress conditions.

**Key words:** *Edwardsiella ictaluri, Plecoglossus altivelis*, subclinical infection, epizootiology, wild fish

The genus *Edwardsiella* (Ewing et al., 1965) in the family Enterobacteriaceae contains two fish pathogens: *E. tarda* and *E. ictaluri*. The genus was first reported under the name of *Paracolobactrum anguillimortiferum* as a pathogen associated with "red disease" in Japanese eel *Anguilla japonica* (Hoshina, 1962). *Edwardsiella tarda* has been shown to cause diseases in a wide variety of cultured freshwater and seawater fishes (Wakabayashi and Egusa, 1973; Evance et al., 2011; Plumb and Hanson, 2011) and is also known as a pathogen of amphibians, reptiles, birds, and mammals, including humans (Sakazaki, 2005). *Edwardsiella ictaluri* was first isolated from diseased channel catfish *Ictalurus punctatus* in the USA in 1976 (Hawke, 1979) and was classified in 1981 (Hawke et al., 1981). Thereafter, *E. ictaluri* has been recognized as the pathogen of enteric septicemia of catfish (ESC) in various cultured and wild catfish species, causing heavy economic losses. Additionally, natural infections with this pathogen have been reported in non-catfish species, including cultured Japanese eel, European sea bass *Dicentrarchus labrax*, rainbow trout *Oncorhynchus mykiss*, wild rudd *Scardinius erythrophthalmus*, and ornamental fishes such as green knife fish *Eigemmannia virescens*, danio *Danio rerio*, and rosy barb *Puntius conchonius* (reviewed in Evance et al., 2011; Plumb and Hanson, 2011). Recently, *E. ictaluri* was isolated from the excrement of wild mink whale *Balaenoptera acutorostrata* (Ogawa et al., 2010).

Ayu *Plecoglossus altivelis*, which is distributed in Japan, Korea, and China, is an amphidromous fish with a 1-year life cycle (Lucas and Baras, 2001), and is one of the most important freshwater fish species in fisheries and recreational fishing in Japan. Ayu matures in autumn and moves downstream to the lower reaches of rivers to spawn, and die. The offspring migrates upstream from coastal areas in spring and grows by feeding on periphyton (algae attached to the riverbed) during summer. Landlocked populations of this species are also present in Lake Biwa in Shiga Prefecture.
the biggest lake in Japan (Fig. 1). Since the natural migration of ayu in Japanese rivers is heavily disturbed by dams and other artificial stream barriers, both fisheries and recreational fishing of ayu require supplementation by artificial release of the juveniles into rivers. Annual releases into rivers are performed by local fishermen's cooperative associations, and include juvenile ayu derived from three sources: hatcheries; natural production in Lake Biwa; and natural production in the coastal areas. Farming of ayu using these juveniles is also popular in western Japan but has suffered from a range of diseases caused by bacterial pathogens belonging to the genera *Vibrio*, *Aeromonas*, *Streptococcus*, *Pseudomonas*, *Flavobacterium*, and *Renibacterium* (Austin and Austin, 2007; Nagai et al., 2008). There have been few reports on disease occurrence with mass mortality in wild ayu populations, where the causative agents include *Vibrio cholerae non-O1* (Muroga et al., 1979; Kiyukia et al., 1992), *Aeromonas hydrophila* (Jo and Onishi, 1980), and *Flavobacterium psychrophilum* (Iida and Mizokami, 1996).

More recently, in 2007 several mass mortality events were recorded in riverine ayu in Tokyo Metropolis, Hiroshima Prefecture, and Yamaguchi Prefecture, Japan; in each case, microbial and pathological examinations implicated *E. ictaluri* as the causative agent (Nagai et al., 2008; Sakai et al., 2008). These reports represent the first records of *E. ictaluri* infection of fish in Japan. Thereafter, this newly emerging disease of river ayu was recorded in 10 or more prefectures west of the Kanto region (Japan Fisheries Resource Conservation Association, personal communication) but without the severe mortality seen in 2007, suggesting that *E. ictaluri* infection in river ayu was decreasing or becoming less virulent. To better characterize the status of *E. ictaluri* infection in river fish, epizootiological investigations were performed from 2008 to 2010, including the screening of apparently healthy fish for the pathogen. The present study was performed in the Gono River in Hiroshima Prefecture, Japan, one of the sites where mortality due to *E. ictaluri* infection was recorded in 2007 (Sakai et al., 2008).

### Materials and Methods

**Fish examined**

Fish were collected at a fixed sampling site in a tributary of the Gono River located in Miyoshi City (Fig. 1). The river has several tributaries in the city. Natural migration of ayu to these tributaries from the sea (across Shimane Prefecture) is very limited due to the presence of a dam; thus, the ayu populations in these rivers depend on annual restocking with juvenile fish. Fish were caught by gill nets from night to early morning and transported (on ice) to the laboratory in Hiroshima University; bacterial isolation was initiated within 1 h of transport. Fish sampling (42–70 fish per time point) was carried out once per month, typically late in the month, from July to November 2008, August to November 2009, and June to November 2010. A total of 723 ayu were examined. Eleven fish species other than ayu, caught at the same place and time, were also screened for bacteria; these other species included Japanese crucian carp *Carassius cuvieri* (n = 10), barbel steed *Hemibarbus barbus* (n = 44), longnose barbel *H. longirostris* (n = 1), largemouth bass *Micropterus salmoides* (n = 9), striped shiner *Pungtungia herzi* (n = 21), pike gudgeon *Pseudogobio esocinus* (n = 3),
Japanese catfish *Silurus asotus* (n = 2), kawa-higai *Sarcochelichthys variegatus variegates* (n = 4), Japanese dace *Trobilodon hakonensis* (n = 48), pale chub (freshwater minnow, *Zacco platypus*, n = 11), and forktail bullhead *Peleobagrus nudiceps* (n = 31). Water temperature data of the river was obtained from the web site of Ministry of Land, Infrastructure, and Transport, Japan (water information system, http://www1.river.go.jp/), which measures water temperature at a monitoring station near the sampling site. The data are shown as the monthly mean of the daily highest temperature, usually recorded between 5 and 6 PM.

In order to identify potential sources of infection, juvenile ayu were collected just before the 2010 release into the Gono River and its tributaries in the city. These juveniles were reared from hatchery-produced larvae in Takehara City (Hiroshima Prefecture), naturally produced larvae caught in Lake Biwa, or naturally produced juveniles caught near the coasts of Miyazaki or Kagoshima Prefecture and transported to the river (Fig. 1). Details of the fish sources are provided in the results section below.

**Isolation and identification of E. ictaluri from fish**

For direct bacterial isolation, trunk kidneys of juvenile or older fish were used as a heavy inoculum on tryptophan-soya agar (TS agar; Nissui) and *Salmonella-Shigella* agar (SS agar; Nissui); following isolation streaking, plates were incubated at 30°C for 48 h. For indirect (selective enrichment culture) bacterial isolation, *Salmonella-Shigella* broth (SS broth) was inoculated with kidney and incubated at 30°C for 48 h; the resulting culture was streaked onto SS agar and *Edwardsoella* isolation medium (EIM) (Shotts and Waltman, 1990), and the plates were incubated at 30°C for 48 h. For organisms derived by either method, *E. ictaluri*-like colonies were picked from the agar plates and screened. Those colonies that were negative for cytochrome oxidase and positive for slide agglutination with a rabbit anti-*E. ictaluri* (anti-PHO744) serum (Hassan et al., 2010) were identified as *E. ictaluri*. Speciation was confirmed using polymerase chain reaction (PCR) with an *E. ictaluri*-specific primer set, EDi (EDi-F: 5′-CAGATGAGCGGATTTCACAG-3′; EDi-R: 5′-CGCGGAATTACATAGAGCC-3′), that targets the upstream region of the fimbrial gene cluster (470 bp) as described previously (Sakai et al., 2009a). The *E. ictaluri* isolates were stocked at −80°C in heart infusion broth (HI broth; Nissui) containing 25% glycerol for further biophysical, biochemical, serological, and genetic characterization.

**Isolation of E. ictaluri-specific bacteriophages from river water**

As evidence for the existence of *E. ictaluri* in the environment, potential *E. ictaluri* phages were isolated from river water on a monthly basis; water was sampled at the same site in the Gono River as that used for fish sampling. Phage isolation was performed using the published enrichment method (Park et al., 2000). A semi-quantitative method was conducted for the estimation of *E. ictaluri* phage concentration using 1, 10, 50, 100, 200, and 400 mL river water. Water samples in duplicate were mixed with an equal volume of double-strength HI broth inoculated with a mixture (approximately 10⁷ CFU/mL) of five isolates of *E. ictaluri* (D4, AH0801, AH0816, PH0744, Oth29) as host bacteria. After overnight incubation under static conditions at 25°C, 1 mL of the culture was centrifuged at 1,500 ×g for 20 min and the supernatant was filtered using a 0.45-μm membrane filter (Advantec). Lytic phages in the filtrate were detected by the spot method using the double agar layer technique (Carlson, 2005). Briefly, 400 μL of each *E. ictaluri* cell suspension at the exponential growth phase were mixed with 3 mL of molten semi-solid agar (TS broth with 0.35% agar, maintained at 50°C), and the mixture was poured onto a TS agar plate. A small amount (20 μL) of the filtrate was inoculated onto the double agar layer plate, and the plate was incubated at 25°C overnight. The quantity of phages in the sampled water was scored on a 6-point scale, where the highest level (Level 6) indicated that phages were isolated even from the smallest water sample (1 mL), and the lowest level (Level 1) indicated that phages were isolated only from the largest water sample (400 mL).

**Characterization of E. ictaluri isolates**

*Edwardsoella ictaluri* isolates obtained between 2008 and 2010 in the present study were subjected to biophysical and biochemical characterization, and compared to previously reported *E. ictaluri* strains isolated from diseased ayu in 2007 and 2008 (Nagai et al., 2008; Sakai et al., 2009a) as well as *E. ictaluri* strains isolated from channel catfish (the type strain JCM1680; ATCC33202) (Hawke et al., 1981) and from striped catfish *Pangasius hypophthalmus* in Indonesia and Vietnam (Yuasa et al., 2003; Hassan et al., 2010). In addition, two strains of *E. tarda*, the type strain JCM1656 (=ATCC15947) and the FK1051 strain isolated from diseased Japanese flounder *Paralichthys olivaceus*, were used for comparison (Table 1). These bacteria were cultured on TS agar at 30°C for 48 h prior to assay. Biophysical and biochemical characterization was performed by the standard methods (MacFaddin, 1980). All tests were performed at 30°C, except for the motility test (at 25°C). Hydrogen sulfide (H₂S) production was tested in triple sugar iron (TSI) agar (Eiken, Japan) and in sulfide-indole-motility (SIM) semisoloid medium (Nissui). Motility was determined in wet mounts with light microscopy and in semisoloid SIM. For serological characterization, two rabbit anti-*E. ictaluri* sera (anti-PHO744 and anti-JCM1680) produced in the previous
study (Hassan et al., 2010) were used in this study. Antigenicity of *E. ictaluri* isolates was compared by a slide agglutination method with 10- and 20-fold diluted antisera, where live cells were used as agglutinogen.

Genetic relatedness among the representative isolates of *E. ictaluri*, including those from striped catfish, was examined by random amplified polymorphic DNA (RAPD) analysis. These assays were performed using Ready-To-Go RAPD Analysis Kit (GE Healthcare) with six primers (P1 to P6), according to the manufacturer’s instructions.

### Infection of fish

Infection experiments were performed using healthy cultured ayu and Japanese catfish, which had been reared at Hiroshima Prefectural Technology Research Institute and at a private farm, respectively, as well as two wild (river-collected) species, forktail bullhead and pale chub. The fish were infected using three *E. ictaluri* isolates (PH0744, AH0816, D4) from ayu. To generate the inocula, bacteria were cultured on TS agar at 30°C for 48 h and suspended in saline at $10^8$ or $10^9$ CFU/mL. Fish were acclimated in 250-L aquaria with flow-through water for 1 week under experimental conditions and injected intraperitoneally (0.1 mL/fish) with each isolate. After challenge, fish were observed in tanks with flow-through water for 10 days. The control fish received injections of sterile saline. Dead fish were collected daily, and isolation of bacteria from the kidney was performed using TS agar. The isolated bacteria were confirmed as *E. ictaluri* by slide agglutination with the anti-PH0744 serum.

### Results

#### Isolation of *E. ictaluri* from river fish

Some of the examined fish (ayu and others), irrespective of the presence of *E. ictaluri*, exhibited hemorrhages or hemorrhagic ulcers on the body surface and internal bloody or clear ascites. However, the majority of the fish displayed no conspicuous clinical signs. *Edwardsiella ictaluri*-like, smooth, circular, slightly punctate colonies, approximately 1 mm in diameter, appeared after 48 h incubation at 30°C on TS agar when using the direct isolation method. These colonies appeared as essentially pure cultures on the plate, typically as a small number of colonies but often as more abundant colonies. When using the indirect isolation method (i.e., after enrichment culture in SS broth), colonies of the same phenotype as above predominated upon plating on SS agar, or appeared as essentially pure cultures upon plating on EIM. In most cases, the direct method using TS or SS agar gave higher isolation rates than the indirect method, and EIM gave higher isolation rates than SS agar in the indirect method. Isolation rates of *E. ictaluri* from the kidney of ayu were 33.2% (98/295) in 2008, 23.6% (54/229) in 2009, and 21.6% (43/199) in 2010. As shown in Table 2, which summarizes the 3-year results, the bacterium was isolated at lower rates in June, July, and August and at...
higher rates in September, October, or November (except for 2009). The highest isolation rate was 68.9% in September 2008; one isolate (AH0816) in 2008 was used as a representative in some of the subsequent investigations. For all 3 years, sexual differentiation of the fish was clearly observed in samplings in September and later, and the ratios of males and females in fish (n = 194) from which the pathogen could be isolated were 48.5% and 35.6%, respectively. In addition, six dead ayu were collected from the river in the August sampling of 2008. *Edwardsiella ictaluri* was isolated as a pure culture from the kidneys of two of these dead fish; one of these isolates was designated D4. Examination of other fish species (n = 2.9 million) from the river ayu in 2008–2010

Table 2. Isolation of *E. ictaluri* from river ayu in 2008–2010

<table>
<thead>
<tr>
<th>Month</th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td></td>
<td>water temp. (°C)</td>
<td>body weight (g)</td>
<td>isolation rate (%)</td>
</tr>
<tr>
<td>June</td>
<td>20.4 ± 1.9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>July</td>
<td>26.6 ± 3.1</td>
<td>60.0 ± 19.9</td>
<td>15.7 (11/70)</td>
</tr>
<tr>
<td>August</td>
<td>26.9 ± 2.6</td>
<td>71.9 ± 12.0</td>
<td>18.3 (11/60)</td>
</tr>
<tr>
<td>September</td>
<td>23.9 ± 2.6</td>
<td>90.5 ± 16.9</td>
<td>68.9 (42/61)</td>
</tr>
<tr>
<td>October</td>
<td>18.8 ± 1.7</td>
<td>1078 ± 33.9</td>
<td>55.6 (25/45)</td>
</tr>
<tr>
<td>November</td>
<td>12.6 ± 2.5</td>
<td>88.6 ± 172</td>
<td>10.2 (9/59)</td>
</tr>
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</table>

*| Monthly mean of the daily highest temperature. | Number of fish positive for *E. ictaluri*/total number of fish examined. | ND, not done.|

biologically and biochemically, with the exception of H₂S production. All isolates were negative for cytochrome oxidase, indole, VP, arginine decarboxylase, phenylalanine deaminase, urease, citrate (Simmons) and malonate utilization, beta-galactosidase, esculin and Tween-80 hydrolysis, DNase, and acid production from sucrose, mannitol, arabinose, and trehalose; all the isolates were positive for catalase, MR, lysine and ornithine decarboxylase, nitrate reduction, and acid production from glucose, fructose, galactose, maltose, man­nose, glycerol, and ribose. The TSI profile was K/A. These characteristics were consistent with the isolates in the previous mortality of wild ayu (Nagai et al., 2008) and with the *E. ictaluri* type strain JCM1680 used as a reference strain. No motility was observed under the light microscope by the wet-mount method, but weak motility was observed in all of the isolates as a form of small budding or ballooning emerging from the inoculated streak in SIM at 25°C. H₂S production was more evident in SIM (67.9% positive) than in TSI (17.1% positive); the type strain was negative in this assay. All isolates were positive in slide agglutination with both 10-fold diluted anti-PH0744 and anti-JCM1680 sera. However, when 20-fold diluted antisera were used, the bacterial isolates reacted only with the anti-PH0744 serum, while JCM1680 remained the only strain to react with the anti-JCM1680 serum.

A preliminary RAPD analysis with primers P1 to P6 was performed using two *E. ictaluri* strains (PH0744, JCM1680) and two *E. tarda* strains (FK1051, JCM1656). The P2 primer showed the widest range of polymorphic bands among the strains examined (data not shown). When we randomly screened 27 strains from ayu and forktail bullhead and analyzed by RAPD with the P2 primer, these isolates exhibited a similar band pattern (Fig. 2A). This RAPD pattern resembled that seen in *E. ictaluri* strains derived from striped catfish in Indonesia, but not the pattern seen in bacteria from Vietnam, JCM1680, and *E. tarda* strains (Fig. 2B).
Subclinical *E. ictaluri* infection of wild ayu


**Infection of fish**

Challenge with strain PH0744 at a dose of $10^{7.1}$ CFU/fish resulted in 100% mortality of ayu both at 25°C and 30°C and 100% mortality of Japanese catfish and forkail bullhead at 25°C, but only 15% mortality of pale chub at 25°C (Table 3). Both the AH0816 and D4 isolates also were virulent to ayu even at a dose of $10^{5.4}$ CFU/fish. Dead ayu displayed exophthalmia, reddening of the head, hemorrhagic ascitic fluid, or hemorrhagic protruded vent, while other fish species did not show any gross clinical signs, except for ascites. *E. ictaluri* was successfully re-isolated from the kidneys of all dead fish. No mortalities were recorded in the control (saline-inoculated) groups.

**Table 3.** Pathogenicity of ayu *E. ictaluri* isolates in four freshwater fish by intraperitoneal injection

<table>
<thead>
<tr>
<th><em>E. ictaluri</em> isolate</th>
<th>Species</th>
<th>Mean (± SD) body weight (g)</th>
<th>Water temp. (± 1°C)</th>
<th>Injection dose (CFU/fish)</th>
<th>No. of fish dead/examined (mortality %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH0744</td>
<td>Ayu</td>
<td>6.9 ± 1.1</td>
<td>25</td>
<td>$10^{7.1}$</td>
<td>24/24 (100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Control</td>
<td>0/25 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$10^{7.1}$</td>
<td>25/25 (100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Control</td>
<td>0/25 (0)</td>
</tr>
<tr>
<td></td>
<td>Japanese catfish</td>
<td>5.9 ± 1.4</td>
<td>25</td>
<td>$10^{7.1}$</td>
<td>18/18 (100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Control</td>
<td>0/20 (0)</td>
</tr>
<tr>
<td></td>
<td>Forktail bullhead</td>
<td>84.2 ± 38.6</td>
<td>25</td>
<td>$10^{7.1}$</td>
<td>3/3 (100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Control</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td></td>
<td>Pale chub</td>
<td>6.0 ± 4.9</td>
<td>25</td>
<td>$10^{7.1}$</td>
<td>3/20 (15)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Control</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>AH0816</td>
<td>Ayu</td>
<td>8.5 ± 0.4</td>
<td>21</td>
<td>$10^{14}$</td>
<td>25/25 (100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$10^{5.4}$</td>
<td>24/24 (100)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Control</td>
<td>0/25 (0)</td>
</tr>
<tr>
<td>D4</td>
<td>Ayu</td>
<td>8.5 ± 0.4</td>
<td>21</td>
<td>$10^{14}$</td>
<td>23/24 (96)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$10^{5.4}$</td>
<td>19/24 (79)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Control</td>
<td>0/25 (0)</td>
</tr>
</tbody>
</table>

*Plecoglossus altivelis  *Silurus asotus  *Peleobagrus nudiceps  *Zacco platypus

Control, saline injection.
Isolation of E. ictaluri phages from river water

_Edwardsiella ictaluri_-lytic phages were readily isolated from river water. Monthly isolation results are shown in Fig. 3, where the quantity of phages was expressed as Level 1 (the lowest) to Level 6 (the highest). Phages could be detected at Level 6 in October to December 2009. Thereafter, the quantity decreased gradually to Level 2 in March and April 2010, and then to Level 1 in May and June 2010. The level increased suddenly to Level 5 in July 2010 and reached Level 6 in August and September of that year. _Edwardsiella ictaluri_ phages were also detected in river sediments at the same sampling site throughout the study period. The phages exhibited myoviral morphology; the host-range was specific for _E. ictaluri_ and not for _E. tarda_ and other fish-pathogenic bacteria (data not shown).

**Discussion**

Investigations performed over 3 successive years revealed that a large number of free-living (riverine) ayu were subclinically or asymptomatically infected with _E. ictaluri_. An _E. ictaluri_ carrier-like state also was confirmed in ayu populations in rivers west of the Kanto region (Tokyo area) through routine monitoring (PCR-based detection) conducted by several prefectural fisheries research institutes (Japan Fisheries Resource Conservation Association, personal communication). These results suggest that _E. ictaluri_ infection is endemic among ayu in Japanese rivers, though serious mass-mortality events have not been seen since the first outbreaks in 2007. As observed in the present study, however, high-level _E. ictaluri_ infection (as evidenced by isolation of abundant colonies) was observed in not a few fish. Therefore, the current subclinical infection state might turn to overt infection and thus result in mortality. Previous studies reported that cultured channel catfish could serve as _E. ictaluri_ carriers, both by experimental and natural infection (Klesius, 1992; Mgolomba and Plumb, 1992; Antonio and Hedrick, 1994); however, with one exception (Chen et al., 1994), there are no reports of subclinical infection or carrier condition in wild populations of fish.

In 2 years (2008, 2010) of the present study, isolation rates of _E. ictaluri_ from river ayu were lower in summer (June to August) and greatly increased in September and October or November, coincident with decreased water temperature; this pattern was less apparent in 2009 (Table 2). The basis of lower infection rates in summer cannot be simply attributed to the bacterium, as it has an optimum growth temperature of 25–30°C (Hawke, 1979; Nagai et al., 2008). This interpretation was supported by the observation (Table 3) that the strain was pathogenic to ayu at 30°C, which is the upper limit of water temperature for ayu. The higher infection rates in fall may reflect lowered immunity of fish after high water temperature in summer and/or physiological disorders associated with sexual maturation. Either of these conditions might induce the pathogen’s invasion and replication in fish, consistent with the previous outbreaks from late August to early October (Sakai et al., 2008). If water temperature stress is a key predisposing factor, then lower infection rates in September and October in 2009 may be due to relatively lower water temperatures in the summer of that year (Table 2). We are not aware of reports on changes in the immune system of ayu during sexual maturation. A similar seasonal occurrence has been described in ESC of cultured channel catfish in Alabama, USA, with the greatest incidence of the disease observed in May–June and September–October, when average water temperatures ranged from 20 to 28°C (Plumb and Hanson, 2011). In experimental infection of channel catfish, _E. ictaluri_ is highly virulent at 25°C water temperature but less virulent at 21°C or lower and 30°C or higher (Francis-Floyd et al., 1987; Baxa-Antonio et al., 1992). Interestingly, there have been almost no mass mortality cases of _E. ictaluri_ infection in cultured ayu. Since underground well water with a constant temperature at 17–18°C throughout the year is generally used for ayu culture, moderate water temperature may be a contributing factor in reducing mortality in cultured ayu. However, temperature modulation of infection does not exactly fit the present case, because ayu are still susceptible to experimental infection of _E. ictaluri_ at 17.5°C (Sakai et al., 2008), suggesting that the current situation does not preclude the occurrence of the disease in ayu aquaculture.

In seeking possible sources of _E. ictaluri_ infection in river ayu, we performed several corollary experiments. Initially, we hypothesized that juvenile ayu released into...
the river were asymptomatically infected with the pathogen. However, *E. ictaluri* was not isolated from the juvenile ayu we examined. Similarly, a variety of ayu populations at larval, juvenile, or adult stages were also suspected, but virtually all examined, except for some populations of naturally produced larvae in Lake Biwa, were negative for *E. ictaluri* (data not shown). Indeed, these *E. ictaluri*-carrying larvae populations became negative for pathogen isolation during the course of their rearing period. Elimination of the bacterium from fish may be due to the use of chemotherapeutics during rearing, since *E. ictaluri* is highly susceptible to many drugs (Waltman and Shotts, 1986), including those licensed for ayu culture in Japan. The use of antibiotics may be another reason why the disease has rarely occurred in cultured ayu. An alternative biotic possibility of the infection source is wild fish species inhabiting the river, since *E. ictaluri* was isolated from forktail bullhead (albeit from only 1 of 31 fish examined). In a PCR-based study (Tensha and Hatama, 2009), the pathogen was detected in Japanese eel and pale chub as well as in forktail bullhead obtained from the Nishiki River (Iwakuni City, Yamaguchi Prefecture), a site that had a fish kill in 2007. This possibility is supported by our observation that forktail bullhead (Bagridae) and another catfish species, Japanese catfish (Siluridae), were susceptible to experimental infection by the *E. ictaluri* isolated from ayu (Table 3). Considering that several non-catfish species are susceptible to *E. ictaluri*, either naturally or experimentally (Evance et al., 2011), we cannot rule out the possibility that these fishes other than ayu are reservoirs or susceptible hosts for *E. ictaluri* under natural conditions.

It has been reported that *E. ictaluri* could survive in pond-bottom mud for extended periods at 18 or 25°C in the absence of other microbes (Plumb and Quinlan, 1986). To consider the possibility of abiotic source for infection, we tried to isolate *E. ictaluri* from river water and sediments; these efforts were unsuccessful, probably due to overgrowth by other microorganisms (data not shown). As an alternative way to demonstrate the presence of *E. ictaluri* in river environments, we isolated *E. ictaluri*-specific phages (Fig. 3). Notably, the phages were abundantly (more than 1 particle/mL) isolated from river water in autumn (October to December), when *E. ictaluri*-carriage rates in ayu increased (Table 2). Phages were consistently detected (albeit at low levels) in winter and early spring (January to March), when ayu completely disappeared from the river, and in early summer (April to June), when ayu reappear in the environment following artificial releases. The number of phages increased again in July to September. This pattern of phage detection suggests that *E. ictaluri* is ubiquitous in the river environment, indicating that environmental *E. ictaluri* is the most probable infection source for ayu. *Edwardsiella ictaluri* can infect channel catfish by several routes (Evance et al., 2011). As reported for *Vibrio anguillarum* or *Pseudomonas plecoglossicida* infection of ayu, both skin and fins are thought to be the portals of entry for those pathogens (Kanno et al., 1989; Sukenda and Wakabayashi, 2001), and possibly for *E. ictaluri*. Once infected, ayu may retain *E. ictaluri* for extended periods, as has been seen in channel catfish (Klesius, 1992; Mgolomba and Plumb, 1992), probably due to the macrophage phagocytosis-resistant nature of this pathogen (Miyazaki and Plumb, 1985; Booth et al., 2006; Russo et al., 2009). The infection cycle might be completed between environmental pathogen (*E. ictaluri*) and susceptible host (ayu or other fishes) in riverine settings.

*Edwardsiella ictaluri* is known as a homogeneous species with regard to the phenotypic and genetic properties (Evance et al., 2011; Plumb and Hanson, 2011). The biophysical and biochemical characteristics of *E. ictaluri* isolated from healthy ayu in the present study were consistent with those of strains isolated from diseased fish (Nagai et al., 2008) and also those of strains isolated from other fish species (Hawke et al., 1981; Waltman et al., 1986; Plumb and Vinitnantharat, 1989), although note should be made of differences in motility and H₂S production. *Edwardsiella ictaluri* is defined as non-motile in *Bergey’s Manual of Systematic Bacteriology* (Sakazaki, 2005); our previous scanning electron microscopy failed to demonstrate flagella in the isolates from diseased ayu (Nagai et al., 2008). However, several literature reports suggest that *E. ictaluri* is weakly motile (Evance et al., 2011). Indeed, among the *E. ictaluri* isolates from ayu described here, motility was difficult to confirm directly by the conventional wet-mount method, but it was confirmed indirectly as swarming in SIM medium. This confirmation is consistent with the results of Newton and Triche (1993), who showed that the degree of motility varied among *E. ictaluri* isolates, with motility greatly increased by repeated passage through semisolid agar; these authors additionally detected peritrichous flagellation by transmission electron microscopy. The isolates described here also varied in H₂S production. Although *E. ictaluri* is negative for H₂S production with TSI medium (Hawke et al., 1981; Plumb and Vinitnantharat, 1989), the production of H₂S has been shown by a lead acetate paper method, whereby 86.2% (n = 109) of the isolates examined were positive for sulfide (Waltman et al., 1986). In the present study, using SIM, H₂S production was evidenced in the isolates at high rates (67.9%, n = 113); sulfide levels were equivalent to those seen in non-motile *E. tarda* strains, but lower than those seen in motile *E. tarda* isolates. Accordingly, a full assessment of H₂S production should be determined for *E. ictaluri* strains from different sources.

Our previous sequence analyses on 16S rDNA, a
heat shock protein gene (dnaJ), and a Type 1 fimbrial gene (effA), demonstrated genetic relatedness among _E. ictaluri_ isolates from diseased ayu (Nagai et al., 2008). RAPD, which is known to provide an efficient assay for polymorphism (Williams et al., 1990), was used to assess the diversity among the _E. ictaluri_ isolates derived in the present study. High similarity by RAPD analysis among the _E. ictaluri_ isolates in Japan indicates that these strains may be clonal, possibly originating from a single source. This hypothesis is supported by the strains’ antigenic homogeneity, which was in turn distinct from the type strain and from _E. ictaluri_ isolates derived from non-Japanese catfish. This hypothesis also is consistent with the results of cross-serum absorption in a previous study (Hassan et al., 2010). Sakai et al. (2009a) deduced the same conclusion from the identity of Japanese _E. ictaluri_ isolates in the amplified-fragment length polymorphism (AFLP) analysis, and furthermore suggested that Japanese _E. ictaluri_ possibly originated from a country or countries in Southeast Asia. In a preliminary experiment using the _E. ictaluri_ phages isolated in the present study, we found that phage sensitivity differs between Japanese and foreign isolates, and that there were some differences in phage sensitivity among the isolates from ayu. These results suggest that phage typing may be useful in elucidating the transmission route of _E. ictaluri_.

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海生甲殻類から分離した病原卵巣類のITS1 領域の塩基配列による同定とアルテミア孵化幼生に対する病原性
村長保勝・佐野文子・畑井嘉司雄
海生甲殻類から分離した病原卵巣類27株について形態分類を行うとともに、対照とした卵巣類6種12株と合わせ
せて、ITS1領域の塩基配列を比較した。その結果、塩基配列に基づく系統関係は形態学的分類に良く一致し
種の同定に有用であることが示唆された。また、9種18株の
病原卵巣についてアルテミアのノウプリウス幼生に対する
浸漬攻撃試験（1×10^5  zosspores/mL, 25℃）によって
病原性的検定を行ったところ、死亡率は供試した卵巣類
によって大きく異なった。
魚病研究. 47 (2), 41-48 (2012)

感染実験からみた病魚細菌のサケ科魚類卵内感染機序
小原昌和・笠井久会・吉水 守
ニジマスおよびアマゴ卵を用いて Flavobacterium
psychrophilum, Renibacterium salmoninarum および
Aeromonas salmonidica の感染実験を行い、卵内感染機
序を検討した。F. psychrophilum は卵の吸水時に卵門から
侵入すると考えられた。F. psychrophilum 感染群は、
汚染水吸水卵よりも卵表面汚染後に吸水させた卵で有意
に高く、成立条件は 10^-5 CFU/mL 以上であった。また、
高濃度の R. salmoninarum で表面汚染した卵においても
卵内感染がみられた。F. psychrophilum または A.
salmonicida 汚染卵を吸水させたところ、F. psychrophilum
は卵内侵入後に増殖したが、A. salmonicida は侵入後数
日で消滅した。
魚病研究. 47 (2), 49-55 (2012)

タイのテラピア養殖場から分離された Aeromonas
hydrophila 多剤耐性株
N. Tipmongkolpip - C. S. del Castillo - 引間順一
T-S. Jung - 近藤秀裕 - 山本貴生 - 宇田 宙
タイのテラピア養殖場の感染魚より55株の A.
hydrophila を分離し、11薬剤の最小発育阻止濃度を調べ
た。その結果、全ての分離株が 1 ～8剤の組合せの耐性
を示し、5剤以上の耐性薬が約半数を占めた。これらの
薬剤耐性株中、1株からABPC, CP, SM, SMMX および
TC の5剤に対して耐性を示す伝達性 R プラスマドを検出
した。このプラスマドは、耐性遺伝子として blaOXA-35,
cat2, aadA1, sul1 および tetA を含んでいた。
魚病研究. 47 (2), 56-63 (2012)

川河アユにおける Edwardsiella ictaluri 不顕性感染
E. S. Hassan - M. M. Mahmoud - 河村信彦 - 永井 崇裕
川口 修 - 飯田悦也 - 清浅 啓 - 中井敬博
2008年から2010年にかけて、広島県下の1川河川におい
て E. ictaluri の保菌調査を実施した。アユは本菌が高
濃度で分離され、特に9月以降の保菌率は高く平
均45.4%であった。アユ以外の魚種では10尾のギフトから分
離されたにすぎず、また魚の由来を問わずおびえた
放流アユ種苗からは本菌はまったく検出されなかった。
一方、E. ictaluri の指標としてのフィアが河川水から周
年にわたって検出されたことから、本菌は河川環境に常
在化し、それが川河アユへの感染源になると考えられた。
魚病研究. 47 (2), 64-73 (2012)

在来マス及びアユに対する Yersinia ruckeri の病原性
坂井 貴光 - 中島 千早 - 伊東静史 - 三輪 理
大迫道久 - 飯田 賢次
ニジマス、イワナ、アマゴ及びヤマメの腹腔内に Y.
ruckeri を 7.1×10^5 CFU/魚体重（g）接種して攻撃した。
各魚種の累積死亡率は、100％、60％、30％、30％であ
り、すべての死亡魚がレッドマス病の症状を示した。
1.5×10^5 ～1.5×10^6 CFU/魚体重（g）で腹腔内接種した
アユの累積死亡率は、0％～87％であった。また、浸漬
攻撃でも高い累積死亡率を観察された。死亡したアユに
サケ科魚類と同様のレッドマス病の症状は見られず、
眼球の突出や出血、腹水の貯留が観察された。
魚病研究. 47 (2), 74-79 (2012)

ポルトガルで養殖ターボットに発生した Streptococcus
parauberis 感染症
M. F. Ramos - J. F. Marques - J. V. Neves - T. Barandela
J.A. Sousa - A. Saraiva - P. N. Rodrigues
2004年5月から8月にかけてポルトガル北部の1養
殖場でターボット（体重 90~1,020 g 水温12-14℃）に
眼瞼突出、背部や鰭基部の出血と浮腫、また病理解剖学的
的には胸腺炎を特徴とする大量死亡が発生した。病魚の
内臓諸器官からグラム陽性の α溶血性球菌が分離され、
それらは生化学的・血清学的性状および遺伝学的性状
（16S rDNA を標的とした PCR）から S. parauberis に同
定された。これはポルトガルにおける本菌感染症の初報
告である。
魚病研究. 47 (2), 80-82 (2012)