

Antibiotic Resistance and Virulence-Associated Gene Profiles of *Edwardsiella tarda* Isolated from Cultured Fish in Japan

Sudu Hakuruge Madusha Pramud Wimalasena¹, Hansani Nilupama Kumari Senarath Pathirana¹, Benthotage Chamara Jayasankha De Silva¹, Sabrina Hossain¹, Emi Sugaya², Toshihiro Nakai³, Gang-Joon Heo^{1,*} 

¹ Chungbuk National University, Veterinary Medical Center and College of Veterinary Medicine, Cheongju, 28644, Korea.

² Fukuyama University, Faculty of Life Science and Biotechnology, Fukuyama, Hiroshima 729-0292, Japan.

³ Hiroshima University, Graduate School of Biosphere Science, Higashi-Hiroshima, Hiroshima 739-8528, Japan.

Article History

Received 15 November 2017

Accepted 19 March 2018

First Online 23 March 2018

Corresponding Author

Tel.: +82.43 2612617

E-mail: gjheo@cbu.ac.kr

Keywords

Edwardsiella tarda

Edwardsiellosis

Antibiotic resistance genes

Virulence gene

Pathogenicity

Phylogenetics

Paralichthys olivaceus

Pagrus major

Abstract

Edwardsiella tarda is one of the most significant bacterial pathogens to fish aquaculture worldwide. Here, we evaluated antibiotic resistance profiles, virulence genotypes and phylogenetic relationship of 30 *Edwardsiella tarda* isolates derived from cultured fish in Japan. The *E. tarda* isolates were phylogenetically (16S rRNA) clustered into two clads. The isolates of clad 1, which were mainly originated from olive flounder *Paralichthys olivaceus* and Japanese eel *Anguilla japonica*, had all of the known 11 virulence genes (*ank*, *katB*, *LuxS*, *citC*, *astA*, *evpP*, *mukF*, *gadB*, *fimA*, *hlyA*, *esa*) for *E. tarda* and mostly one or two of three aminoglycoside resistance genes, i.e. *aac(6)-Ib*, *armA* and *strA-strB*, if exists. In contrast, the isolates of clad 2, which were mainly originated from red seabream *Pagrus major* and black seabream *Acanthopagrus schlegelii*, lacked four (*ank*, *katB*, *LuxS*, *citC*) of the 11 virulence genes and, eight of them harbored mainly a β -lactam resistance gene (*bla_{CTX-M}*). In infection experiments with zebrafish, representative isolates of the clad 1 showed higher virulence than those of the clad 2. These results reveal that the virulence profiles may be applicable for prediction of pathogenicity in fish. Phylogenetic analysis revealed that *E. tarda* isolates derived from olive flounder and red seabream are ancestor differentiated.

Introduction

In recent years, disease outbreaks caused by bacterial pathogens have affected fish farming all over the world. Edwardsiellosis caused by *Edwardsiella tarda* has been reported worldwide in economically important fish species, including Japanese eel *Anguilla japonica*, red seabream *Pagrus major*, olive flounder *Paralichthys olivaceus*, channel catfish *Ictalurus punctatus*, Asian catfish *Clarias batrachus* and turbot *Scophthalmus maximus* (Alcaide, Herraiz, & Esteve, 2006; Castro *et al.*, 2016; Kim *et al.*, 2014; Xu & Zhang, 2014).

At present, 17 antibiotics (antibacterial drugs) are approved for aquaculture use and from them 5 drugs are used to treat edwardsiellosis in Japan (Ministry of Agriculture, Forestry and Fisheries http://www.maff.go.jp/j/syouan/suisan/suisan_yobo/in

[dex.html](#)). However, the massive use of antibiotics in commercial fish culture increases the selective pressure on microbial flora and promotes the natural increase in antibiotic resistance. Not only resistant bacteria can multiply after an antibiotic has suppressed sensitive bacteria, but also they can transfer the resistance genes bearing plasmids and transposons to other bacteria that have never been exposed to the antibiotics (Aoki, Arai, & Egusa, 1977; Aoki & Kitao, 1981; Aoki, Kitao, & Fukudome, 1989; Stock & Wiedemann, 2001; Wei *et al.*, 2011). Furthermore, previous studies have characterized that antibiotic resistance is more common in pathogenic compared to commensal organisms and is often linked to virulence factors of the pathogen (Beceiro, Tomás, & Bou, 2013; Yu, Cho, Kim, & Kang, 2012).

Pathogenicity of *E. tarda* depends on the number of virulence factors including the ability to resist serum

killing (Iida and Wakabayashi, 1983), to invade epithelial cells (Janda *et al.*, 1991; Ling, Wang, Xie, Lim, & Leung, 2000), to resist phagocyte-mediated killing (Srinivasa Rao, Lim, & Leung, 2003), and to produce dermatotoxins, adhesins and hemolysins for disseminating infection (Hirono, Tange, & Aoki, 1997). Virulence of *E. tarda* is believed to be a multifactorial process, but it has not yet been completely understood. The pathogenic strains of *E. tarda* have virulence genes which might be absent in non-pathogenic strains (Castro *et al.*, 2016; Srinivasa Rao *et al.*, 2003). Now, many virulence-related genes in *E. tarda* have been cloned and reported, which include catalase (*katB*), type III secretion system (TTSS) (*ast*), TTSS regulator (*esrB*), type VI secretion system (T6SS) extracellular apparatus (*evp*), putative killing factor (*mukF*), fimbrial operon (*fimA*), glutamate decarboxylase (*gadB*), citrate lyase (*citC*), hemolysins (*hlyA*), ankyrin like proteins (*ank*), and quorum sensing system (*luxS*) (Kim *et al.*, 2014; Okuda, Takeuchi, & Nakai, 2014; Srinivasa Rao *et al.*, 2003). Some studies have been conducted to evaluate the pathogenicity of *E. tarda* using the zebrafish *Danio rerio* as the animal model (Kim *et al.*, 2014; Okuda *et al.*, 2014).

However, the majority of the studies on *E. tarda* have examined either the antibiotic resistance (Lo, Lee, Wang, & Kuo, 2014; Wei *et al.*, 2011) or the pathogenicity (Castro *et al.*, 2016; Okuda *et al.*, 2014; Srinivasa Rao *et al.*, 2003) and thus very little information is currently available concerning both antibiotic resistance and pathogenicity of *E. tarda* strains (Kim *et al.*, 2014; Yu *et al.*, 2012). In this study, we investigated the antibiotic susceptibility, antibiotic resistance and virulence-associated gene profiles, phylogenetic diversity, and pathogenicity to zebrafish on *E. tarda* isolates derived from different cultured fish in Japan.

Materials and Methods

Bacterial Isolates

A total of 30 *E. tarda* isolates, which were derived from four different fish species (15 red seabream *Pagrus major*, 10 olive flounder *Paralichthys olivaceus*, 4 Japanese eel *Anguilla japonica*, 1 black seabream *Acanthopagrus schlegelii*) at commercial farms in Japan between 1980 and 2014, were used for this study (Table 1). *E. tarda* strains were isolated from kidney or liver of diseased (dead or moribund) fish, when mass mortality occurred. Bacteria were routinely grown at 25°C on tryptic soy agar (MB Cell, USA) and in tryptic soy broth (TSB) (MB Cell). Stock cultures were maintained at -80°C as suspensions in supplemented 10% skim milk (MB Cell) containing 25% (vol/vol) glycerol.

16S rRNA Gene Sequencing and Phylogenetic Analysis

All *E. tarda* isolates were grown overnight at 30°C in TSB and genomic DNA was extracted using the Exgene Cell SV kit (GeneAll, Korea) following the manufacturer's protocol. The 16S rRNA gene sequencing was performed using universal primers 12F and 1492R. The amplicons were purified using Exgene PCR SV (GeneAll) purification kit and sequenced at Cosmogenetech Co, Ltd. (Seoul, Korea). Taxonomic identification of the DNA sequences was performed using BLAST option in GenBank database (<http://blast.ncbi.nlm.nih.gov/>).

Based on the 16S rRNA gene sequences, a neighbor-joining phylogenetic tree was obtained with 1000 bootstrap replications. For the analysis, four and two published 16S rRNA sequences data of *E. tarda* and *E. ictaluri*, respectively, and a sequence data of *Aeromonas hydrophila* (Figure 1) were obtained from the GenBank database and MEGA6 sequence analyzing software was used for aligning and construction of the phylogenetic tree.

Antimicrobial Susceptibility Test

The antimicrobial susceptibility of the isolates was examined by the disc-diffusion method with 16 antimicrobial agents: ampicillin (10 µg), cefotaxime (30 µg), ceftiofur (30 µg), tetracycline (15 µg), colistin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), trimethoprim-sulfamethoxazole (25 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), streptomycin (10 µg), imipenem (10 µg), gentamicin (10 µg), kanamycin (30 µg), aztreonam (30 µg) and nitrofurantoin (300 µg). Testing was confirmed by duplicating and the resistance profiles (resistant, intermediate, or susceptible) were assigned using criteria described by Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2015). *Escherichia coli* ATCC 25922 was used as the reference strain.

Detection of Antibiotic Resistance Genes

All the isolates were tested by PCR assays to detect the genetic determinants associated with resistance to aminoglycosides [*strA-strB*, *aac(3)-IIa*, *armA*, *aphA1-IAB* and *aac(6')-Ib*], β-lactams (*bla_{TEM}*, *bla_{SHV}*, *bla_{OXA}* and *bla_{CTX-M}*), and quinolones (*qnrA*, *qnrB* and *qnrS*) (Chung, Yi, Kim, Kim, & Shin, 2017; Hu *et al.*, 2013). PCR amplifications were conducted in 20 µL volumes consisting of 10 µL of Quick Taq® HS DyeMix (TOYOBO, Japan), 1 µL of 10 pmol/µL each primer, 1 µL of the template and 7 µL of the PCR grade water under standard conditions. The PCR products were analyzed by electrophoresis on 2% (wt/vol) agarose gels. Positive controls were implemented with previously characterized enterobacterial strains that harbored the corresponding genes (Chung *et al.*, 2017). The presence

Table 1. *Edwardsiella tarda* isolates used in this study

Bacterial strain	Host fish	Year	Location (Prefecture)	Reference
NE8003	Olive flounder	1980	Nagasaki	Okuda et al. (2007)
NUF82	Olive flounder	1984	Nagasaki	Okuda et al. (2007)
NUF251	Olive flounder	1986	Nagasaki	Okuda et al. (2007)
PE113	Olive flounder	2001	Ehime	Okuda et al. (2007)
PE210	Olive flounder	2001	Ehime	-
E01-21	Olive flounder	2001	Ehime	-
E01-33	Olive flounder	2001	Ehime	Okuda et al. (2007)
E01-37	Olive flounder	2001	Ehime	Okuda et al. (2007)
FH-9104	Olive flounder	1991	Hiroshima	-
FK1051	Olive flounder	2001	Hiroshima	-
REF001	Red seabream	1996	Mie	Okuda et al. (2007)
REF146	Red seabream	1996	Mie	Okuda et al. (2007)
REF179	Red seabream	2001	Mie	Okuda et al. (2007)
MEE0203	Red seabream	2002	Mie	-
MEE0301	Red seabream	2003	Mie	-
MEE0309	Red seabream	2003	Mie	-
E01-14	Red seabream	2001	Ehime	Okuda, Kiriya, Yamanoi, and Nakai (2008)
E01-17	Red seabream	2001	Ehime	Okuda et al., (2009)
E01-40	Red seabream	2001	Ehime	-
EhiRS10-1	Red seabream	2011	Ehime	-
EhiRS12-1	Red seabream	2011	Ehime	-
EhiRS12-2	Red seabream	2011	Ehime	-
EY01	Red seabream	2014	Ehime	-
EY02	Red seabream	2014	Ehime	-
Yama-S	Red seabream	-	Ehime	-
SU28	Japanese eel	1980	Shizuoka	-
SU44	Japanese eel	1980	Shizuoka	-
SU53	Japanese eel	1980	Shizuoka	-
SU57	Japanese eel	1980	Shizuoka	-
No. 1	Black porgy	2003	Hiroshima	-

of *cr* variant in *aac(6)-Ib* gene was investigated by sequencing and BLAST comparison with GenBank (<http://blast.ncbi.nlm.nih.gov/>).

Detection of Virulence-Associated Genes

The known 11 virulence-associated genes (*ank*, *katB*, *luxS*, *citC*, *astA*, *evpP*, *mukF*, *gadB*, *fimA*, *hlyA*, *esa*) of *E. tarda* were detected by PCR amplification using previously reported primers (Kim *et al.*, 2014; Li, Ni, Liu, & Lu, 2011) and under the above-described conditions.

Experimental Infection

Wild type zebrafish (weighed 0.28±0.1 g) were obtained from a commercial aquarium in Seoul. During the acclimatization period, fish were fed with commercial diet for 3 times a day (4% of body weight/day), and maintained in automated water circulation system at 28±1°C with 12/12 h light/dark cycle. For injection challenge, representative four *E. tarda* isolates were selected randomly from distinct virulence genotypes; two isolates (FH-9104 and E01-21) harboring eleven virulence genes and two isolates

(MEE0309 and EY02) harboring seven virulence genes. The inoculum was prepared from the culture in TSB at 28°C for 24 h. Experimental fish (n=15) were given an intraperitoneal injection with 10 µL of each bacterial suspension containing 1 x 10² to 1 x 10⁶ CFU/fish. Fish (n=15) in control group were similarly injected with sterile phosphate-buffered saline (PBS, pH 7.4) instead of bacteria. Mortalities were recorded twice daily for one week. Disease signs of moribund and dead fish were observed, and dead fish were removed from the experimental tanks for bacteriological examination. The mean LD₅₀ was determined using the simplified method of Reed and Muench (1938).

Results

Phylogenetic Comparison of 16S rRNA Sequences

Phylogenetic tree derived from the 16S rRNA gene sequences of the present 30 *E. tarda* isolates and the reference strains is illustrated in Figure 1. Neighbor-joining phylogenetic tree indicated two distinct clads. The present *E. tarda* isolates from olive flounder, Japanese eel, one isolate (E01-14) from red seabream and three *E. tarda* reference strains (LTB-4 and

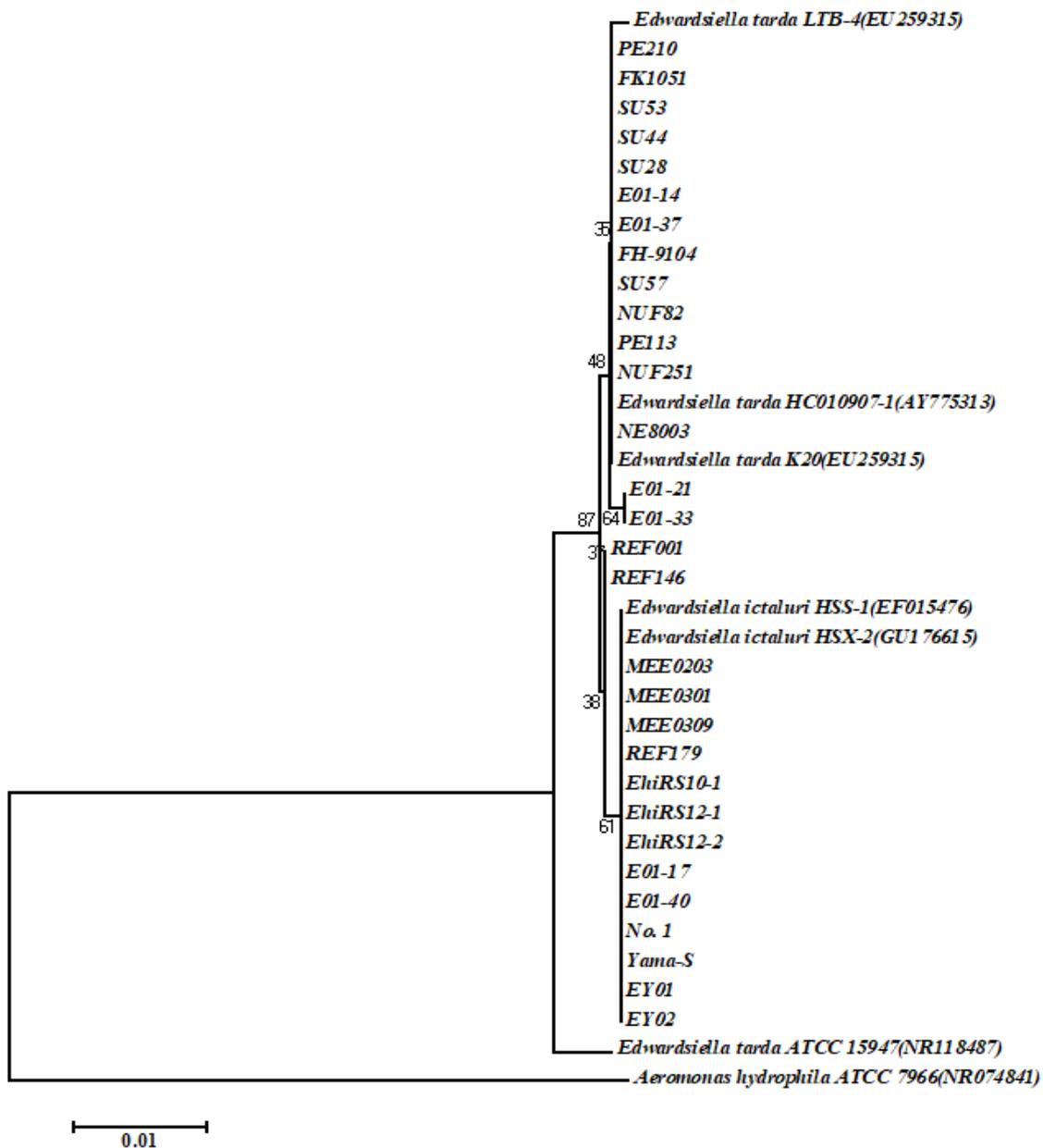


Figure 1. Phylogenetic analysis of the 16S rRNA genes from *Edwardsiella tarda* isolates. The tree was generated based on nucleotide sequences using the maximum-likelihood model in MEGA 6 software. The numbers below the branches indicate the 1000 bootstrap values.

HC010907 from turbot *Scophthalmus maximus*, and K20 from Japanese eel *Anguilla japonica*) comprised the clad 1, and the remaining *E. tarda* isolates from red seabream except E01-14 and one isolate (No.1) from black seabream comprised the clad 2. It should be noted that *E. tarda* isolates belonging to clad 2 were closely related to *E. ictaluri* strains (HSS-1 and HSX-2) which were originated from yellow catfish *Pelteobagrus fulvidraco* (Liu, Li, Zhou, Wen, & Ye, 2010).

Antibiotic Susceptibility

Antibiotic resistance profiles of the *E. tarda* isolates to 16 antibiotics are summarized in Table 2. All or most of isolates were susceptible to nitrofurantoin (F),

cefoxitin (FOX), tetracycline (T), chloramphenicol (C), nalidixic acid (NAL), ciprofloxacin (CIP) and imipenem (IPM). In contrast, all isolates were resistant to colistin (CL) and erythromycin (E), and six or more isolates were resistant to ampicillin (AMP), cefotaxime (CTX), trimethoprim-sulfamethoxazole (SXT) and aztreonam (ATM), while a few isolates showed resistant/intermediate profiles to streptomycin (S), gentamycin (G) and kanamycin (K).

Distribution of Antibiotic Resistance Genes

The aminoglycoside resistance genes, *aac(6')-Ib*, *armA* and *strA-strB*, were detected in 11, 3 and 1, respectively, from 30 *E. tarda* isolates. The isolates

Table 2. Antibiotic resistance, antibiotic resistance genes, virulence genes, and 16S rRNA clads of 30 *Edwardsiella tarda* isolates

<i>E. tarda</i> isolates	Host fish	Antibiotic resistance ^a	Antibiotic resistance genes	Virulence genes										16S rRNA clads		
				<i>ank</i>	<i>katB</i>	<i>luxS</i>	<i>citC</i>	<i>ast</i>	<i>evp</i>	<i>mukF</i>	<i>gadB</i>	<i>fimA</i>	<i>hly</i>		<i>Esa</i>	
NE8003	Olive flounder	CL, E	<i>armA</i>	+	+	+	+	+	+	+	+	+	+	+	+	1
NUF82	Olive flounder	CL, E, AMP, CTX	<i>armA</i>	+	+	+	+	+	+	+	+	+	+	+	+	1
NUF251	Olive flounder	CL, E, AMP, CTX	<i>aac(6')-Ib</i>	+	+	+	+	+	+	+	+	+	+	+	+	1
PE113	Olive flounder	CL, E, AMP, SXT, ATM, S, G, K, NAL	<i>aac(6')-Ib</i>	+	+	+	+	+	+	+	+	+	+	+	+	1
PE210	Olive flounder	CL, E, AMP, SXT, ATM, S, C	<i>strA-strB</i>	+	+	+	+	+	+	+	+	+	+	+	+	1
E01-21	Olive flounder	CL, E	-	+	+	+	+	+	+	+	+	+	+	+	+	1
E01-33	Olive flounder	CL, E, AMP, CTX, FOX	<i>aac(6')-Ib</i>	+	+	+	+	+	+	+	+	+	+	+	+	1
E01-37	Olive flounder	CL, E	<i>aac(6')-Ib</i>	+	+	+	+	+	+	+	+	+	+	+	+	1
FH-9104	Olive flounder	CL, E, AMP, CTX, T	-	+	+	+	+	+	+	+	+	+	+	+	+	1
FK1051	Olive flounder	CL, E, SXT, S, T	<i>aac(6')-Ib</i>	+	+	+	+	+	+	+	+	+	+	+	+	1
E01-14	Red seabream	CL, E, AMP, ATM	<i>aac(6')-Ib</i>	+	+	+	+	+	+	+	+	+	+	+	+	1
SU44	Japanese eel	CL, E	<i>aac(6')-Ib</i>	+	+	+	+	+	+	+	+	+	+	+	+	1
SU53	Japanese eel	CL, E, AMP, CTX, SXT, G	<i>aac(6')-Ib</i>	+	+	+	+	+	+	+	+	+	+	+	+	1
SU57	Japanese eel	CL, E	<i>aac(6')-Ib, armA</i>	+	+	+	+	+	+	+	+	+	+	+	+	1
SU28	Japanese eel	CL, E	-	-	-	-	-	+	+	+	+	+	+	+	+	1
REF001	Red seabream	CL, E, SXT	-	-	-	-	-	+	+	+	+	+	+	+	+	2
REF146	Red seabream	CL, E, SXT	-	-	-	-	-	+	+	+	+	+	+	+	+	2
REF179	Red seabream	CL, E, AMP	<i>bla_{CTX-M}</i>	-	-	-	-	+	+	+	+	+	+	+	+	2
MEE0203	Red seabream	CL, E, AMP	<i>aac(6')-Ib, bla_{CTX-M}</i>	-	-	-	-	+	+	+	+	+	+	+	+	2
MEE0301	Red seabream	CL, E, AMP, ATM	<i>bla_{CTX-M}</i>	-	-	-	-	+	+	+	+	+	+	+	+	2
MEE0309	Red seabream	CL, E	-	-	-	-	-	+	+	+	+	+	+	+	+	2
E01-17	Red seabream	CL, E, AMP	<i>bla_{CTX-M}</i>	-	-	-	-	+	+	+	+	+	+	+	+	2
E01-40	Red seabream	CL, E, AMP	<i>aac(6')-Ib, bla_{CTX-M}</i>	-	-	-	-	+	+	+	+	+	+	+	+	2
EhiRS10-1	Red seabream	CL, E, AMP, CTX, ATM	<i>bla_{CTX-M}</i>	-	-	-	-	+	+	+	+	+	+	+	+	2
EhiRS12-1	Red seabream	CL, E, AMP, SXT	<i>bla_{CTX-M}</i>	-	-	-	-	+	+	+	+	+	+	+	+	2
EhiRS12-2	Red seabream	CL, E, AMP	-	-	-	-	-	+	+	+	+	+	+	+	+	2
EY01	Red seabream	CL, E, AMP, ATM, NAL	<i>qnrS</i>	-	-	-	-	+	+	+	+	+	+	+	+	2
EY02	Red seabream	CL, E, SXT	-	-	-	-	-	+	+	+	+	+	+	+	+	2
Yama-S	Red seabream	CL, E, AMP	<i>bla_{CTX-M}</i>	-	-	-	-	+	+	+	+	+	+	+	+	2
No. 1	Black seabream	CL, E	-	-	-	-	-	+	+	+	+	+	+	+	+	2

^a Antibiotic resistance was designated using breakpoints described by the Clinical Laboratory Standards Institute (CLSI, 2015). Antibiotics; FOX= cefoxitin (30 µg), T= tetracycline (15 µg), C= chloramphenicol (30 µg), NAL= nalidixic acid (30 µg), CL= colistin (10 µg), E= erythromycin (15 µg), AMP= ampicillin (10 µg), CTX= cefotaxime (30 µg), SXT= trimethoprim-sulfamethoxazole (25 µg), ATM= aztreonam (30 µg), S= streptomycin (10 µg), G= gentamicin (10 µg) and K= kanamycin (30 µg)

harboring these aminoglycoside resistance genes were mostly olive flounder and Japanese eel, except three isolates (E01-14, MEE0203, E01-40) from red seabream which harbors *aac(6')-Ib* gene. Among the tested β-lactam resistance genes, only the *bla_{CTX-M}* gene was detected in 8 isolates from red seabream. The quinolone resistance gene (*qnrS*) was detected in one isolate (EY01) from red seabream (Table 2).

Distribution of Virulence Genes

Two distinct virulence genotypes were identified in the studied *E. tarda* isolates. Fourteen *E. tarda* isolates, including all the isolates from olive flounder, three isolates (SU44, SU53, SU57) from Japanese eel and one isolate (E01-14) from red seabream, were positive for all 11 tested virulence genes. The rest 16 *E. tarda* isolates, including 14 isolates from red seabream, one isolate (SU28) from Japanese eel and one isolate (No.1) from black seabream, were positive for seven virulence genes (*astA*, *evpP*, *mukF*, *gadB*, *fimA*, *hlyA*, *esa*) (Table 2).

LD₅₀ Values

Following the challenge infection with four representative *E. tarda* isolates, disease manifestations appeared in fish between day 1 and 5 post-challenge, showing reduced activity, anorexia, convulsions, and death. Mortalities of fish were different between two different virulence genotypes. Two isolates (FH-9104, E01-21) of the virulence genotype harboring all the tested virulence genes showed LD₅₀ values as 4.8×10³ and 2.6×10³ CFU/fish, respectively. Two isolates

(MEE0309, EY02) of the virulence genotype harboring seven virulence genes showed LD₅₀ values as 7.6×10⁴ and 5.4×10⁴ CFU/fish, respectively.

Discussion

In the present study, all of the *E. tarda* isolates derived from cultured fish in Japan were resistant to the colistin and erythromycin. *E. tarda* is considered as intrinsically resistant to colistin and macrolides (Stock & Wiedemann, 2001). However, several studies reported some colistin-sensitive *E. tarda* strains (Michael & Abbott, 1993; Stock & Wiedemann, 2001). The resistant isolates were also found at high frequency for the β-lactams (ampicillin, cefotaxime) and at low frequency for the aminoglycosides (streptomycin, gentamicin, and kanamycin). Previous studies have reported ampicillin-, streptomycin- or kanamycin-resistant *E. tarda* which were originated from cultured fish in Japan (Aoki & Kitao, 1981; Aoki *et al.*, 1989). Wei *et al.*, (2011) reported that 28% and 11% of *E. tarda* isolates from freshwater fish in Malaysia were resistant to ampicillin and kanamycin, respectively. Although only a few isolates were resistant to tetracycline or chloramphenicol in our study, the appearance of *E. tarda* resistant to these antibiotics has been frequently reported in Japanese aquaculture (Aoki *et al.*, 1977; Aoki & Kitao, 1981; Furushita *et al.*, 2003). However, antibiotic resistance and resistant determinants in *E. tarda* strains have not been given enough consideration in Japan. The majority of the studies on this bacterium have examined the pathogenicity while giving a seldom value to the antibiotic resistance (Miyazaki, & Egusa,

1976; Matsuyama, Kamaishi, Ooseko, Kurohara, & Lida, 2005; Okuda *et al.*, 2014). Further studies are needed to explore the prevalence and trend of antimicrobial resistance in *E. tarda* from Japanese aquaculture.

The β -lactams, aminoglycoside and quinolone resistance genes targeted in our study were selected based on the abundance of resistant *E. tarda* strains to the antibiotics. Only one resistance gene (*bla_{CTX-M}*) to the β -lactams was detected in eight isolates from red seabream. All of these isolates were resistant to ampicillin and one isolate (EhiRS10-1) was resistant to cefotaxime (a β -lactam), but other five cefotaxime-resistant isolates (NUF82, NUF251, E01-33, FH-9104, SU53) did not carry the *bla_{CTX-M}* gene. Previous studies have also reported the mismatch phenomenon between antimicrobial resistance phenotypes and genotypes of bacteria (Lou, Liu, Zhang, Pan, & Zhao, 2016; Wimalasena, De Silva, Hossain, Pathirana, & Heo, 2017; Zhang, Zhang, & Fang, 2009). It is interesting that aminoglycoside resistance gene *aac(6)-Ib* was most frequently detected in the present *E. tarda* isolates and most of the isolates exhibited high multidrug resistance. Since aminoglycoside resistance genes are often located on large plasmids that carry genes encoding resistance to other antimicrobial agents (Huang *et al.*, 2012; Yu *et al.*, 2012), plasmids may play a role to antibiotic resistance of fish-pathogenic bacteria in Japanese aquaculture. According to the available few reports on antibiotic resistance determinants in *E. tarda* from aquaculture, this bacterium consistently harbored tetracycline and chloramphenicol resistance genes but not β -lactams and aminoglycoside resistance genes (Yoo, Huh, Eun, Lee, & Jeong, 2003; Jin *et al.*, 2004).

Recently, studies on pathogenicity mechanism of *E. tarda* have made great progress, and many virulence factors and relevant genes have been identified (Okuda *et al.*, 2014; Srinivasa Rao *et al.*, 2003). Seven of 11 known virulence genes were detected in all the present *E. tarda* isolates. However, the other four virulence genes (*ank*, *katB*, *luxS* and *citC*) were harbored only in 14 *E. tarda* isolates mainly from olive flounder and Japanese eel. The previous study showed that *ank*, *katB* and *citC* genes were present only in virulent strains of *E. tarda* (Nakamura *et al.*, 2013; Srinivasa Rao *et al.*, 2003). Kim *et al.*, (2014) reported less distribution of *ank* and *luxS* genes in *E. tarda* isolated from Japanese eel in Korea, compared with other virulence genes including *fimA*, *evpP*, *esa* and *astA*. Our result, i.e. lack of *ank*, *katB*, *luxS* and *citC* in the majority of the present *E. tarda* isolates from red seabream, suggests the involvement of virulence factors other than these four genes in the pathogenesis to red seabream.

In this study, the adult zebrafish was used as a model organism to evaluate the virulence of *E. tarda* isolated from cultured fish. Previous studies also have used zebrafish as a model organism for pathogenicity test because this species is easy to cultivate and maintain and its whole genome has been sequenced

(Okuda *et al.*, 2014; Wang, Yan, Wang, Ding, & Li, 2012). According to the pathogenicity test results, the LD₅₀ values of isolates harboring 11 virulence genes were approximately ten times lower than those of isolates harboring seven virulence genes. This correlation between virulence gene profiles and virulence to zebrafish in *E. tarda* isolates suggests that the virulence gene profiles might be one factor which can be used for the prediction of pathogenicity of *E. tarda*. Similar findings in which the number of virulence genes of *E. tarda* isolates were correlated with the pathogenicity to blue gourami *Trichogaster trichopterus* (Srinivasa Rao *et al.*, 2003) and zebrafish (Wang *et al.*, 2012) were reported. In infection experiments using olive flounder and red seabream, Matsuyama *et al.* (2005) reported a different host-specificity between *E. tarda* (so-called typical strain) from olive flounder and that (atypical strain) from red seabream. As mentioned above, it seems that the host-specificity of *E. tarda* is associated with difference of virulence genes.

Phylogenetic analysis of the 16S rRNA gene sequences showed that the present *E. tarda* isolates were separated into two clads (Figure 1). All the isolates originating from olive flounder and Japanese eel were clustered into clad 1, while all the isolates from red seabream with one exception were clustered into clad 2, indicating source associated phylogenetic clustering in *E. tarda* isolates from Japanese cultured fish. This suggests that *E. tarda* isolates derived from olive flounder and red seabream are differentiated from a common ancestor in response to niche adaptation (Yang *et al.*, 2012). Furthermore, it is interesting that the clad 2 isolates were clustered with *E. ictaluri* (HSX-2 reference strain originated from yellow catfish in China). A comparative phylogenomic study revealed that *E. tarda* could be classified into two genotypes (EdwGI, EdwGII) and EdwGI is more closely related to *E. ictaluri* than to *E. tarda* (Yang *et al.*, 2012). Further pathological and genetic studies are required to explain the host-specificity of fish-pathogenic *E. tarda* and *E. ictaluri*.

References

- Alcaide, E., Herraiz, S., & Esteve, C. (2006). Occurrence of *Edwardsiella tarda* in wild European eels *Anguilla anguilla* from Mediterranean Spain. *Diseases of Aquatic Organisms*, 73(1), 77–81.
- Aoki, T., Arai, T., & Egusa, S. (1977). Detection of R plasmids in naturally occurring fish-pathogenic bacteria, *Edwardsiella tarda*. *Microbiology and Immunology*, 21(2), 77–83.
- Aoki, T., & Kitao, T. (1981). Drug resistance and transferable R plasmids in *Edwardsiella tarda* from fish culture ponds. *Fish Pathology*, 15(3–4), 277–281.
- Aoki, T., Kitao, T., & Fukudome, M. (1989). Chemotherapy against infection with multiple drug resistant strains of *Edwardsiella tarda* in cultured eels. *Fish Pathology*, 24(3), 161–168.
- Beceiro, A., Tomás, M., & Bou, G. (2013). Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? *Clinical Microbiology*

- Reviews, 26(2), 185–230.
<https://dx.doi.org/10.1128/CMR.00059-12>
- Castro, N., Osorio, C. R., Buján, N., Fuentes, J. C., Rodríguez, J., Romero, M., ... Magariños, B. (2016). Insights into the virulence-related genes of *Edwardsiella tarda* isolated from turbot in Europe: genetic homogeneity and evidence for vibrioferrin production. *Journal of Fish Diseases*, 39(5), 565–576.
<https://dx.doi.org/10.1111/jfd.12389>
- Chung, T., Yi, S., Kim, B., Kim, W., & Shin, G. (2017). Identification and antibiotic resistance profiling of bacterial isolates from septicemic soft-shelled turtles (*Pelodiscus sinensis*). *Veterinární Medicina*, 62(3), 169–177. <https://dx.doi.org/10.17221/65/2016-VETMED>
- CLSI. (2015). Performance standards for antimicrobial susceptibility testing of bacteria isolated from aquatic animals. second informational supplement VET03/VET04-S2. Wayne, PA, USA.
- Furushita, M., Shiba, T., Maeda, T., Yahata, M., Kaneoka, A., Takahashi, Y., & Ohta, M. (2003). Similarity of tetracycline resistance genes isolated from fish farm bacteria to those from clinical isolates. *Applied and Environmental Microbiology*, 69(9), 5336–5342.
<https://dx.doi.org/10.1128/AEM.69.9.5336-5342.2003>
- Hirono, I., Tange, N., & Aoki, T. (1997). Iron-regulated haemolysin gene from *Edwardsiella tarda*. *Molecular Microbiology*, 24(4), 851–856.
<https://dx.doi.org/10.1046/j.1365-2958.1997.3971760.x>
- Hu, X., Xu, B., Yang, Y., Liu, D., Yang, M., Wang, J., & Ma, X. (2013). A high throughput multiplex PCR assay for simultaneous detection of seven aminoglycoside-resistance genes in Enterobacteriaceae. *BMC Microbiology*, 13(1), 58.
- Huang, X.-Z., Frye, J. G., Chahine, M. A., Glenn, L. M., Ake, J. A., Su, W., & Lesho, E. P. (2012). Characteristics of plasmids in multi-drug-resistant Enterobacteriaceae isolated during prospective surveillance of a newly opened hospital in Iraq. *PLoS ONE*, 7(7), e40360.
<https://dx.doi.org/10.1371/journal.pone.0040360>
- Iida, T., & Wakabayashi, H. (1983). Bactericidal reaction by the alternative pathway of fish complement. *Fish Pathology*, 18, 77-83. (in Japanese with English abstract)
- Janda, J. M., Abbott, S. L., Kroske-Bystrom, S., Cheung, W. K., Powers, C., Kokka, R. P., & Tamura, K. (1991). Pathogenic properties of *Edwardsiella* species. *Journal of Clinical Microbiology*, 29(9), 1997–2001.
- Jin Jun, L., Bum Jeong, J., Huh, M.-D., Chung, J.-K., Choi, D., Lee, C.-H., & Jeong, H.D. (2004). Detection of tetracycline-resistance determinants by multiplex polymerase chain reaction in *Edwardsiella tarda* isolated from fish farms in Korea. *Aquaculture* 240(1-4), 89–100.
<https://dx.doi.org/10.1016/j.aquaculture.2004.07.025>
- Kim, K. I., Kang, J. Y., Park, J. Y., Joh, S. J., Lee, H. S., & Kwon, Y. K. (2014). Phenotypic traits, virulence-associated gene profile and genetic relatedness of *Edwardsiella tarda* isolates from Japanese eel *Anguilla japonica* in Korea. *Letters in Applied Microbiology*, 58(2), 168–176.
<https://dx.doi.org/10.1111/lam.12172>
- Li, J., Ni, X. D., Liu, Y. J., & Lu, C. P. (2011). Detection of three virulence genes *alt*, *ahp* and *aerA* in *Aeromonas hydrophila* and their relationship with actual virulence to zebrafish: Extracellular factor genes and virulence in *A. hydrophila*. *Journal of Applied Microbiology*, 110(3), 823–830.
<https://dx.doi.org/10.1111/j.1365-2672.2011.04944.x>
- Ling, S. H. M., Wang, X. H., Xie, L., Lim, T. M., & Leung, K. Y. (2000). Use of green fluorescent protein (GFP) to study the invasion pathways of *Edwardsiella tarda* in in vivo and in vitro fish models. *Microbiology*, 146(1), 7–19.
- Liu, J. Y., Li, A. H., Zhou, D. R., Wen, Z. R., & Ye, X. Z. P. (2010). Isolation and characterization of *Edwardsiella ictaluri* strains as pathogens from diseased yellow catfish *Pelteobagrus fulvidraco* (Richardson) cultured in China. *Journal of Aquaculture and Research*, 41, 1835–1844.
- Lo, D. Y., Lee, Y. J., Wang, J. H., & Kuo, H. C. (2014). Antimicrobial susceptibility and genetic characterisation of oxytetracycline-resistant *Edwardsiella tarda* isolated from diseased eels. *Veterinary Record*, 175(8), 203–203.
<https://dx.doi.org/10.1136/vr.101580>
- Lou, Y., Liu, H., Zhang, Z., Pan, Y., & Zhao, Y. (2016). Mismatch between antimicrobial resistance phenotype and genotype of pathogenic *Vibrio parahaemolyticus* isolated from seafood. *Food Control*, 59, 207–211.
<https://dx.doi.org/10.1016/j.foodcont.2015.04.039>
- Matsuyama, T., Kamaishi, T., Ooseko, N., Kurohara, K., & Iida, T. (2005). Pathogenicity of motile and non-motile *Edwardsiella tarda* to some marine fish. *Fish Pathology*, 40(3), 133–135.
- Michael, J., & Abbott, S. L. (1993). Infections associated with the genus *Edwardsiella*: the role of *Edwardsiella tarda* in human disease. *Clinical Infectious Diseases*, 17(4), 742–748.
- Miyazaki, T., & Egusa, S. (1976). Histopathological studies of edwardsiellosis of the Japanese eel (*Anguilla japonica*). Suppurative interstitial nephritic form. *Fish Pathology*, 11: 33-43.
- Nakamura, Y., Takano, T., Yasuike, M., Sakai, T., Matsuyama, T., & Sano, M. (2013). Comparative genomics reveals that a fish pathogenic bacterium *Edwardsiella tarda* has acquired the locus of enterocyte effacement (LEE) through horizontal gene transfer. *BMC Genomics*, 14(1), 642.
- Okuda, J., Murayama, F., Yamanoi, E., Iwamoto, E., Matsuoka, S., Nishibuchi, M., & Nakai, T. (2007). Base changes in the *fliC* gene of *Edwardsiella tarda*: possible effects on flagellation and motility. *Diseases of Aquatic Organisms*, 76, 113–121.
- Okuda, J., Kiriya, M., Yamanoi, E., & Nakai, T. (2008). The type III secretion system-dependent repression of NF- κ B activation to the intracellular growth of *Edwardsiella tarda* in human epithelial cells. *FEMS Microbiology Letters*, 283, 9–14.
- Okuda, J., Kiriya, M., Suzaki, E., Kataoka, K., Nishibuchi, M., & Nakai, T. (2009). Characterization of proteins secreted from a Type III secretion system of *Edwardsiella tarda* and their roles in macrophage infection. *Diseases of Aquatic Organisms*, 84, 115–121.
- Okuda, J., Takeuchi, Y., & Nakai, T. (2014). Type III secretion system genes of *Edwardsiella tarda* associated with intracellular replication and virulence in zebrafish. *Diseases of Aquatic Organisms*, 111(1), 31–39.
<https://dx.doi.org/10.3354/dao02763>
- Reed, L. J., & Muench, H. (1938). A simple method of estimating fifty percent end points. *American Journal of Hygiene*, 27, 493–497.
- Srinivasa Rao, P. S., Lim, T. M., & Leung, K. Y. (2003). Functional genomics approach to the identification of virulence genes involved in *Edwardsiella tarda* pathogenesis. *Infection and Immunity*, 71(3), 1343–1351.
<https://dx.doi.org/10.1128/IAI.71.3.1343-1351.2003>

- Stock, I., & Wiedemann, B. (2001). Natural antibiotic susceptibilities of *Edwardsiella tarda*, *E. ictaluri*, and *E. hoshinae*. *Antimicrobial Agents and Chemotherapy*, 45(8), 2245–2255.
<https://dx.doi.org/10.1128/AAC.45.8.2245-2255.2001>
- Wei, L. S., Musa, N., Seng, C. T., Shazili, N. A. M., Wee, W., Musa, N., & Wahid, M. E. A. (2011). Antibigram and plasmid profiling from *Edwardsiella tarda* isolated from freshwater fish in east coast Malaysia. *Journal of Sustainability Science and Management*, 6(1), 19–27.
- Wang, X., Yan, M., Wang, Q., Ding, L., & Li, F. (2012). Identification of *Edwardsiella tarda* isolated from duck and virulence genes detection. *African Journal of Microbiology Research*, 6(23).
<https://dx.doi.org/10.5897/AJMR11.1604>
- Wimalasena, S. H. M. P., De Silva, B. C. J., Hossain, S., Pathirana, H. N. K. S., & Heo, G.-J. (2017). Prevalence and characterization of quinolone resistance genes in *Aeromonas* species isolated from pet turtle in Korea. *Journal of Global Antimicrobial Resistance*.
<https://dx.doi.org/10.1016/j.jgar.2017.06.001>
- Xu, T., & Zhang, X.-H. (2014). *Edwardsiella tarda*: an intriguing problem in aquaculture. *Aquaculture*, 431, 129–135.
<https://dx.doi.org/10.1016/j.aquaculture.2013.12.001>
- Yang, M., Lv, Y., Xiao, J., Wu, H., Zheng, H., Liu, Q., ... Wang, Q. (2012). *Edwardsiella* comparative phylogenomics reveal the new intra/inter-species taxonomic relationships, virulence evolution and niche adaptation mechanisms. *PLoS ONE*, 7(5), e36987.
<https://dx.doi.org/10.1371/journal.pone.0036987>
- Yoo, M.H., Huh, M.-D., Kim, E., Lee, H.-H., & Do Jeong, H. (2003). Characterization of chloramphenicol acetyltransferase gene by multiplex polymerase chain reaction in multidrug-resistant strains isolated from aquatic environments. *Aquaculture*, 217(1-4), 11–21.
- Yu, J. E., Cho, M. Y., Kim, J., & Kang, H. Y. (2012). Large antibiotic-resistance plasmid of *Edwardsiella tarda* contributes to virulence in fish. *Microbial Pathogenesis*, 52(5), 259–266.
<https://dx.doi.org/10.1016/j.micpath.2012.01.006>
- Zhang, X.-X., Zhang, T., & Fang, H. H. P. (2009). Antibiotic resistance genes in water environment. *Applied Microbiology and Biotechnology*, 82(3), 397–414.
<https://dx.doi.org/10.1007/s00253-008-1829-z>