Journal of Coastal Life Medicine

journal homepage: www.jclmm.com

Document heading doi:10.12980/JCLM.2.2014JCLM-2014-0005 

# *Edwardsiella tarda* and *Aeromonas hydrophila* isolated from diseased Southern flounder (*Paralichthys lethostigma*) are virulent to channel catfish and Nile tilapia

Julia W. Pridgeon[1*], Phillip H. Klesius[1], Gregory A. Lewbart[2], Harry V. Daniels[3], Megan Jacob[2]

[1]*Aquatic Animal Health Research Unit, USDA-ARS, 990 Wire Road, Auburn, AL 36832, United States of America*

[2]*College of Veterinary Medicine, North Carolina State University, 1060 William Moore Drive, Raleigh, NC 27607, United States of America*

[3]*Department of Applied Ecology, North Carolina State University, Raleigh, NC 27695, United States of America*

PEER REVIEW

**Peer reviewer**

Dr. Xingjiang MU (John Mu), Auburn University, Auburn, Alabama, USA.
Tel: +86-13918668951
E-mail: xjmu@shou.edu.cn

**Comments**

This is a valuable research work in which authors have demonstrated *E. tarda* could be the primary pathogen that caused the disease outbreak in the Southern flounder in the summer of 2012 and these bacteria isolated from diseased Southern flounder are virulent to channel catfish and Nile tilapia. This research results suggest that the size of fish might have an effect on their susceptibility to pathogens.
Details on Page 343

ABSTRACT

**Objective:** To identify bacteria isolated from diseased Southern flounder and determine whether they are virulent to channel catfish and Nile tilapia.
**Methods:** Gram-negative bacteria isolates were recovered from five tissues of diseased Southern flounder (*Paralichthys lethostigma*). The isolates were subjected to biochemical and molecular identification followed by virulence study in fish.
**Results:** Based on biochemical analysis, the 25 isolates were found to share homologies with either *Edwardsiella tarda* (*E. tarda*) or *Aeromonas hydrophila* (*A. hydrophila*). Based on sequencing results of partial 16S rRNA gene, 15 isolates shared 100% identities with the 16S rRNA sequence of previously identified *E. tarda* strain TX1, whereas the other 10 isolates shared 100% identities with the 16S rRNA sequence of previously identified *A. hydrophila* strain An4. When healthy fish were exposed to flounder isolate by intracoelomic injection, the $LD_{50}$ values of flounder isolate *E. tarda* to channel catfish or Nile tilapia [(10±2) g] were $6.1\times10^4$ and $1.1\times10^7$ CFU/fish, respectively, whereas that of flounder isolate *A. hydrophila* to channel catfish and Nile tilapia were $1.4\times10^7$ and $5.6\times10^7$ CFU/fish, respectively.
**Conclusions:** This is the first report that *E. tarda* and *A. hydrophila* isolated from diseased Southern flounder are virulent to catfish and tilapia.

KEYWORDS
*Edwardsiella tarda*, *Aeromonas hydrophila*, Southern flounder, Pathogen, Virulence

## 1. Introduction

Gram-negative bacteria *Aeromonas hydrophila* (*A. hydrophila*) and *Edwardsiella tarda* (*E. tarda*) are known bacterial pathogens to many fish species, including Japanese eel (*Anguilla japonica*)[1], European eel (*Anguilla anguilla*)[2], Japanese flounder (*Paralichthys olivaceus*)[3], koi carp (*Cyprinus carpio*)[4], and channel catfish (*Ictalurus punctatus*)[5]. In addition, both *A. hydrophila* and *E. tarda* could be pathogenic to humans[6,7].

Due to the fact that bacteria can survive well in the aquatic environment independent of their hosts, bacterial diseases have become major impediments to aquaculture, especially when water temperature is warm[8]. In the summer of 2012, mass mortality of Southern flounder was observed in North Carolina, USA, with infected fish showing loss of pigmentation, exophthalmia, opacity of the eyes, swelling of the abdominal surface, and rectal hernia. The role of pathogenic bacteria in this disease outbreak was not known. In addition, information on whether bacteria

*Corresponding author: Julia W. Pridgeon, Aquatic Animal Health Research Unit, USDA-ARS, 990 Wire Road, Auburn, AL 36832, USA.
Tel: 334-887-3741
Fax: 334-887-2983
E-mail: Julia.Pridgeon@ars.usda.gov
Foundation Project: Supported by the USDA/ARS CRIS project #6420-32000-024-00D.

Article history:
Received 23 Jan 2014
Received in revised form 10 Feb, 2nd revised form 21 Feb, 3rd revised form 2 Mar 2014
Accepted 23 Apr 2014
Available online 28 May 2014

isolated from marine species (such as Southern flounder) could be virulent to fresh water species (such as channel catfish and Nile tilapia) is scarce. Therefore, the objectives of this study were: 1) to isolate and identify bacteria from tissues of diseased Southern flounder; 2) to determine whether any of the bacteria isolated from diseased Southern flounder are virulent to channel catfish and Nile tilapia.

## 2. Materials and methods

### 2.1. Bacteria isolation and culture conditions

After mortality was observed in North Carolina in the summer of 2012, five diseased moribund Southern flounder [*Paralichthys lethostigma* (*P. lethostigma*)] were collected, freshly frozen, and sent to the Aquatic Animal Health Research Laboratory, United States Department of Agriculture-Agricultural Research Service (USDA-ARS) within 24 h on ice. A sterile loop was used to inoculate the tissue samples onto tryptic soy agar (TSA) plates. After overnight growth at (28±1) °C, all colonies appeared white. A total of 25 colonies (five from each fish) were then randomly picked and re-streaked onto TSA plates to obtain single colonies. Single colony culture grown in tryptic soy broth was then subjected to biochemical and molecular identification. Glycerol stock (10% glycerol) of each isolate was prepared in tryptic soy broth and stored at −80 °C.

### 2.2. Gram staining, oxidase test, and API 20 NE test

Gram staining was performed with Gram staining kit and reagents followed the instruction of the manufacturer (Becton Dickinson, Franklin Lakes, NJ, USA). Oxidase test was performed by adding bacterial smear to filter paper containing BactiDrop oxidase reagent (Remel, Lenexa, KS, USA). Color development was observed within 1 min. API 20 NE bacterial identification was performed according to manufacturer's instruction (bioMérieux, Durham, NC, USA).

### 2.3. Microbial identification using fatty acid methyl ester (FAME) profiling

FAME profiling was performed according to manufacturer's instructions (MIDI Labs, Newark, DE, USA). Briefly, overnight bacterial cultures (25-30 mg) were transferred to 13 mm×100 mm glass tubes. Bacterial cells were saponified in 3.75 mol/L NaOH in 50% methanol for 30 min, followed by methylation with 3.25 mol/L HCl in methanol for 10 min. FAMEs were then mixed with hexane and methyl tert-butyl ether (1:1) for 10 min. After brief centrifugation, the top phase was collected and mixed with 0.3 mol/L NaOH to remove any free fatty acids and residual extraction solvent. After brief centrifugation, the top phase was removed and subjected to gas chromatography using Agilent 6850 GC system (Agilent Technology, Santa Clara, CA, USA). FAME profiles were then compared to FAME profiles deposited in the standard RCLN or RFISH library and the similarity indices were then calculated by Sherlock Library Search (MIDI, Newark, DE, USA). An exact match of the fatty acid makeup of an unknown sample with that of a known library entry would result in a similarity index of 1 000. Samples with a similarity of 0.500 or higher with a separation of 0.100 between the first and second choice are considered good library comparison.

### 2.4. Genomic DNA extraction, polymerase chain reaction (PCR), and sequencing of 16S rRNA

Genomic DNA was extracted from each bacterial isolate using DNeasy kit (Qiagen, Valencia, CA, USA). All DNAs were quantified on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE, USA). Universal 16S forward primer (16S-F) 5′-AGA GTT TGA TCM TGG CTC AG-3′ and universal 16S reverse primer 5′-AAG GAG GTG WTC CAR CC-3′ were used to amplify 16S rRNA partial gene. PCR was performed in a 25 μL mixture consisting of 12.5 μL of 2 × Taq PCR Master Mix (Qiagen, Valencia, CA, USA), 9.5 μL of nuclease-free $H_2O$, 1 μL of genomic DNA (10 ng/μL), 1 μL of forward primer (5 μmol/L), and 1 μL of reverse primer (5 μmol/L). PCR was carried out in a Biometra T Gradient thermocycler (Biometra, Goettingen, Germany). PCR reaction conditions consisted of an initial denaturation step at 94 °C for 5 min followed by 35 cycles of 45 seconds at 94 °C, 45 seconds at 50 °C, and 2 min at 72 °C, followed by a final extension of 10 min at 72 °C. PCR products were analyzed by 1% agarose gel by electrophoresis. Individual single bands were excised and purified using gel purification kit according to the manufacturer's protocol (Qiagen, Valencia, CA, USA). Purified PCR products were sequenced using 16S-F primer at the USDA-ARS Mid South Genomic Laboratory (Stoneville, MS, USA) on an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

### 2.5. Sequence analysis

Sequences were analyzed using the National Center for Biotechnology Information (NCBI) BLAST program to search for sequence identities. Multiple sequence alignment for partial 16S rRNA sequences was performed using T-coffee method[9,10].

### 2.6. Virulence to channel catfish or Nile tilapia by intracoelomic injection

Prior to molecular identification, five Southern flounder *E. tarda* isolates and three Southern flounder *A. hydrophila*

isolates identified by FAME were randomly selected and exposed to Nile tilapia [(10 or 30±2) g] (20 fish/tank; duplicate tanks for each isolate). For 30 g tilapia, the injection dose was about $1.0\times10^8$ CFU/fish. For 10 g tilapia, two doses ($1.0\times10^7$ and $1.0\times10^8$ CFU/fish) were used. Post molecular identification, sequencing results of partial 16S rRNA gene revealed that the 15 isolates of *E. tarda* shared 100% identities (*E* value=0) with the 16S rRNA sequence of *E. tarda* strain TX1 (GenBank EF467363), whereas the rest 10 isolates of *A. hydrophila* shared 100% identities (*E* value=0) with the 16S rRNA sequence of *A. hydrophila* strain An4 (GenBank FJ386959). Therefore, only two representative isolates (isolate #1 representing *E. tarda* whereas isolate #20 representing *A. hydrophila*) were chosen to determine the $LD_{50}$ values of *E. tarda* or *A. hydrophila* to healthy channel catfish by intracoelomic injection. After overnight incubation of isolate #1 or #20 at (28±1) °C, the average number (CFU/mL) was calculated. Five different doses (ranging from $1\times10^2$ to $1\times10^8$ CFU/fish) that killed 0% to 100% fish was administered to anesthetized (100 mg/L of tricaine methanesulfonate) channel catfish [(10.0±2.5) g] or Nile tilapia [(10.0±2.2) g] through intracoelomic injection (20 fish per tank, duplicate tanks for each dose). After exposing fish to isolate #1 or isolate #20, mortalities were recorded daily for 14 d post exposure. The presence of bacteria in dead fish was determined by culturing anterior kidney samples on TSA plates followed by API 20 NE biochemical test.

### 2.7. Statistical analysis

Cumulative mortality were analyzed with SigmaStat 3.5 software (Systat Software, Inc, Point Richmond, CA, USA) using student's *t*-test. Significance level was defined as $P<0.05$. PoloPlus probit and logit analysis software (LeOra Software, Petaluma, CA, USA) was used to calculate $LD_{50}$ values. Virulence of *E. tarda* and *A. hydrophila* was considered significantly different when the 95% confidence intervals of $LD_{50}$ values failed to overlap ($P\leqslant0.05$).

## 3. Results

### 3.1. Collection of bacterial isolates from diseased Southern flounder

Diseased Southern flounder showed loss of pigmentation, exophthalmia, opacity of the eyes, swelling of the abdominal surface, and rectal hernia (Figure 1). All five diseased fish had growth on TSA plates. A total of 25 colonies were randomly picked and subjected to biochemical and molecular identification.

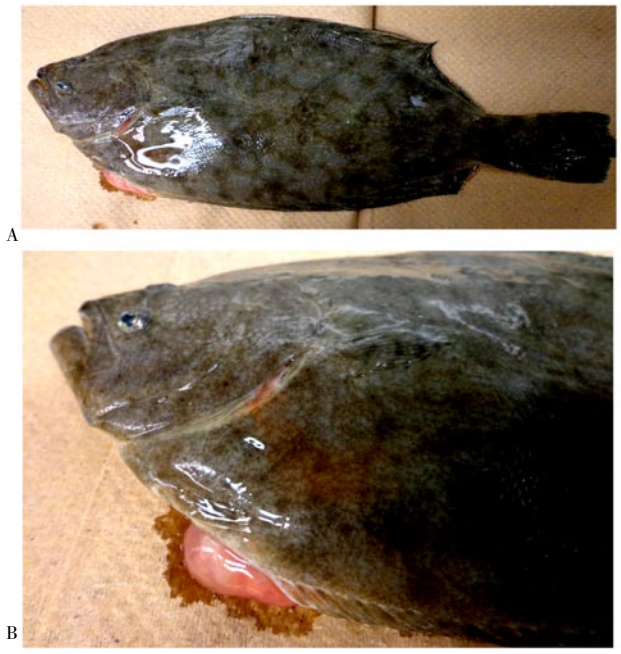

**Figure 1.** Diseased Southern flounder infected by both *E. tarda* and *A. hydrophila*.
A: diseased fish showing loss of pigmentation; B: diseased fish showing exophthalmia, opacity of the eyes, abdominal distension, and rectal hernia.

### 3.2. Gram staining, oxidase test, and API 20 NE test

All 25 isolates were Gram-negative. Of the 25 isolates, 15 were oxidase negative whereas 10 were oxidase positive. Based on API 20 NE test results, of the 25 isolates, 15 shared 99% homologies with *E. tarda* whereas 10 shared 90% homologies with *A. hydrophila*.

### 3.3. FAME analysis of the bacterial isolates

FAME analysis revealed that 15 isolates shared similar profiles with *E. tarda* deposited at the RFISH database, with first choice similarity indices ranging from 0.520 to 0.617. The 15 isolates had five major fatty acid peaks (Figure 2A). The biggest peak was 16:0 fatty acid at retention time 2.754 min, which accounted for 30% of the total FAME response. The second biggest peak was summed feature 3 (16:1w7c/16:1w6c) at retention time 2.702 min, which had 23% of the total response. The third (18:1w7c at retention time 3.339 min) and fourth (14:0 at retention time 2.133 min) fatty acid peak accounted for 11.8% and 10.8% of the total FAME, respectively (Figure 2A). The fifth major fatty acid peak was summed feature 2 (12:0 aldehyde, 16:1 iso I/14:0 3OH at retention time 2.600), which accounted for 9.4% of total FAME response (Figure 2A).

FAME profiling also revealed that 10 isolates shared similar profiles with *A. hydrophila* deposited at the RCLN database, with first choice similarity indices ranging from 0.652 to 0.712. The 10 isolates had three major fatty acid

peaks (Figure 2B). The biggest peak was summed feature 3 (16:1w7c/16:1w6c) at retention time 2.702 min, which had 37% of the total response (Figure 2B). The second biggest peak was 16:0 at retention time 2.754 min, which had 22% of the total response. The third major peak was 18:1w7c at retention time 3.338 min, which accounted for 16% of the total FAME (Figure 2B).

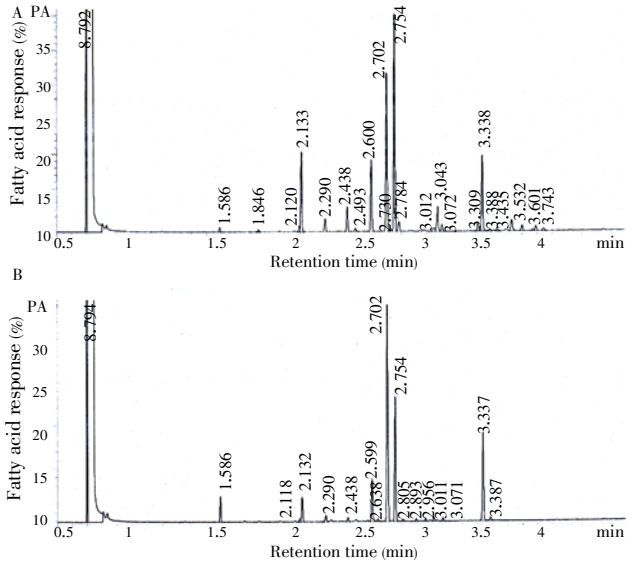


**Figure 2.** Fatty acid methyl ester profiles of bacterial isolates cultured from diseased yellow perch.
A: representative FAME profile of the 15 isolates identified as *E. tarda*; B: representative FAME profile of the 13 isolates identified as *A. hydrophila*.

### *3.4. PCR and sequencing results*

Based on sequencing results of partial 16S rRNA gene, the 15 isolates of *E. tarda* identified by FAME shared 100% identities (*E* value=0) with the 16S rRNA sequence of previously identified *E. tarda* strain TX1 (GenBank EF467363) (Figure 3), whereas the rest 10 isolates of *A. hydrophila* identified by FAME shared 100% identities (*E* value=0) with the 16S rRNA sequence of previously identified *A. hydrophila* strain An4 (GenBank FJ386959) (Figure 4). T-coffee multiple sequence alignment for partial 16S rRNA sequences obtained in this study revealed that the 15 isolates of *E. tarda* shared 100% identities with each other. Similarly, the 10 isolates of *A. hydrophila* shared 100% identities with each other.

### *3.5. Virulence of Southern flounder isolate of E. tarda or A. hydrophila to Nile tilapia*

Results of the virulence of eight randomly selected Southern flounder bacterial isolates to Nile tilapia were summarized in Table 1. At dose of $1.0\times10^8$ CFU/fish, all eight bacterial isolates killed 100% Nile tilapia at size of 10 g (Table 1). However, at dose of about $1.0\times10^7$ CFU/fish, the five isolates of *E. tarda* killed 70% to 80% tilapia at size of 10 g, whereas the three isolates of *A. hydrophila* killed 8% to 13% tilapia at similar size (10 g) (Table 1). When 30 g Nile tilapia were exposed to the eight isolates by injection dose of $1.0\times10^8$ CFU/fish, the five isolates of *E. tarda* killed 70% to 78% fish, whereas the three isolates of *A. hydrophila* killed 5% to 8% fish (Table 1).

```
Query  1    GCCTTAAACATGCAAGTCGAGCGGTAGCAGGGAGAAAGCTTGCTTTCTCCGCTGACGAGC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  13   GCCTTAAACATGCAAGTCGAGCGGTAGCAGGGAGAAAGCTTGCTTTCTCCGCTGACGAGC  72
Query  61   GGCGGACGGGTGAGTAATGTCTGGGGATCTGCCTGATGGAGGGGGATAACTACTGGAAAC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  73   GGCGGACGGGTGAGTAATGTCTGGGGATCTGCCTGATGGAGGGGGATAACTACTGGAAAC  132
Query  121  GGTAGCTAATACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  133  GGTAGCTAATACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCA  192
Query  181  TCAGATGAACCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGAT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  193  TCAGATGAACCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGAT  252
Query  241  CCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  253  CCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCT  312
Query  301  ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  313  ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGC  372
Query  361  GTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTAGGGAGGAAGGTGTGAACGT  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  373  GTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGTAGGGAGGAAGGTGTGAACGT  432
Query  421  TAATAGCGCTCACAATTGACGTTACCTACAGAAGAAGCACCGGCTAACTCCGTGCCAGCA  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  433  TAATAGCGCTCACAATTGACGTTACCTACAGAAGAAGCACCGGCTAACTCCGTGCCAGCA  492
Query  481  GCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCA  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  493  GCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCA  552
Query  541  GGCGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATCCAAGAC  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  553  GGCGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATCCAAGAC  612
Query  601  TGGCAAGCTAGAGTCTCGTAGAGGGAGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAG  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  613  TGGCAAGCTAGAGTCTCGTAGAGGGAGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAG  672
Query  661  AGATCTGGAGGAATACCGGTGGCGAAGGCGGCCTCCTGGACGAAGACTGACGCTCAGGTG  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  673  AGATCTGGAGGAATACCGGTGGCGAAGGCGGCCTCCTGGACGAAGACTGACGCTCAGGTG  732
Query  721  CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTC  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  733  CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTC  792
Query  781  GATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGAAGCTAACGCGTTAAATCGACCGCC  840
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  793  GATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGAAGCTAACGCGTTAAATCGACCGCC  852
Query  841  TGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGT  900
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  853  TGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGT  912
Query  901  GGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACAT  954
            ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  913  GGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACAT  966
```

**Figure 3.** BlastN search results of the partial 16S rRNA sequence of the Southern flounder isolate of *E. tarda*.
Query: partial 16S rRNA sequence of Southern flounder isolate of *E. tarda*; Sbjct: *E. tarda* strain TX1 16S rRNA gene, partial sequence (EF467363).

```
Query 1   GGGCAGGCCTAACAATGCAAGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGG 60
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 8   GGGCAGGCCTAACAATGCAAGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGG 67
Query 61  CGAGCGGCGGACGGGTGAGTAATGCCTGGGAAATTGCCCAGTCGAGGGGGATAACAGTTG 120
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 68  CGAGCGGCGGACGGGTGAGTAATGCCTGGGAAATTGCCCAGTCGAGGGGGATAACAGTTG 127
Query 121 GAAACGACTGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGACCTTCGGGCCTTG 180
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 128 GAAACGACTGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGACCTTCGGGCCTTG 187
Query 181 CGCGATTGGATATGCCCAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCG 240
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 188 CGCGATTGGATATGCCCAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCG 247
Query 241 ACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGA 300
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 248 ACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGA 307
Query 301 CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCAT 360
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 308 CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCAT 367
Query 361 GCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTCA 420
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 368 GCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTCA 427
Query 421 GTAGCTAATATCTGCTGACTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGC 480
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 428 GTAGCTAATATCTGCTGACTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGC 487
Query 481 CAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC 540
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 488 CAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC 547
Query 541 ACGCAGGCGGTTGGATAAG 559
          ||||||||||||||||||
Sbjct 548 ACGCAGGCGGTTGGATAAG 566
```

**Figure 4.** BlastN search results of the partial 16S rRNA sequence of the Southern flounder isolate of *A. hydrophila*.

Query: partial 16S rRNA sequence of Southern flounder isolate of *A. hydrophila*; Sbjct: *A. hydrophila* strain An 4 16S rRNA gene, partial sequence (FJ386959).

**Table 1**

Virulence of *E. tarda* or *A. hydrophila* to 10 g or 30 g Nile tilapia.

| Treatment | | Injection dose (CFU/fish) | Mortality (%) |
|---|---|---|---|
| To 10 g tilapia | Control | 0 | 0±0 |
| | *E. tarda* isolate #1 | $1.0\times10^8$ | 100±0 |
| | *E. tarda* isolate #5 | $1.0\times10^8$ | 100±0 |
| | *E. tarda* isolate #7 | $1.0\times10^8$ | 100±0 |
| | *E. tarda* isolate #10 | $1.0\times10^8$ | 100±0 |
| | *E. tarda* isolate #12 | $1.0\times10^8$ | 100±0 |
| | *A. hydrophila* isolate #19 | $1.0\times10^8$ | 100±0 |
| | *A. hydrophila* isolate #20 | $1.0\times10^8$ | 100±0 |
| | *A. hydrophila* isolate #23 | $1.0\times10^8$ | 100±0 |
| | *E. tarda* isolate #1 | $1.0\times10^7$ | 80±0 |
| | *E. tarda* isolate #5 | $1.0\times10^7$ | 70±0 |
| | *E. tarda* isolate #7 | $1.0\times10^7$ | 80±0 |
| | *E. tarda* isolate #10 | $1.0\times10^7$ | 75±7 |
| | *E. tarda* isolate #12 | $1.0\times10^7$ | 70±0 |
| | *A. hydrophila* isolate #19 | $1.0\times10^7$ | 13±4 |
| | *A. hydrophila* isolate #20 | $1.0\times10^7$ | 10±0 |
| | *A. hydrophila* isolate #23 | $1.0\times10^7$ | 8±4 |
| To 30 g tilapia | Control | 0 | 0±0 |
| | *E. tarda* isolate #1 | $1.0\times10^8$ | 75±7 |
| | *E. tarda* isolate #5 | $1.0\times10^8$ | 70±7 |
| | *E. tarda* isolate #7 | $1.0\times10^8$ | 75±7 |
| | *E. tarda* isolate #10 | $1.0\times10^8$ | 75±0 |
| | *E. tarda* isolate #12 | $1.0\times10^8$ | 78±4 |
| | *A. hydrophila* isolate #19 | $1.0\times10^8$ | 5±0 |
| | *A. hydrophila* isolate #20 | $1.0\times10^8$ | 8±4 |
| | *A. hydrophila* isolate #23 | $1.0\times10^8$ | 8±4 |

Data are expressed as mean±SD.

### 3.6. Virulence of flounder isolate of *E. tarda* or *A. hydrophila* to 10 g channel catfish

Virulence of the Southern flounder bacterial isolates to channel catfish are summarized in Figure 5. At injection dose of $1.0\times10^8$ CFU/fish, all channel catfish were killed. At dose of $1.0\times10^7$ CFU/fish, *E. tarda* killed 100% catfish, whereas *A. hydrophila* killed 25% to 45% catfish (Figure 5A). At injection dose of $1.0\times10^6$ CFU/fish, *E. tarda* killed 100% catfish, whereas *A. hydrophila* killed 0% catfish (Figure 5B).

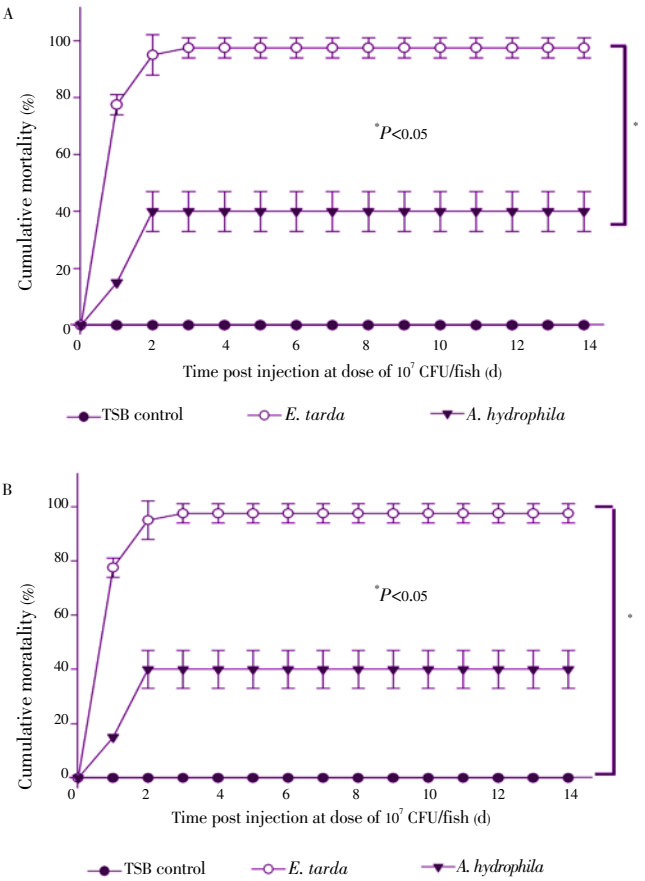


**Figure 5.** Cumulative mortality of 10 g channel catfish after exposure to the Southern flounder isolate of *E. tarda* or *A. hydrophila* by intracoelomic injection.

A: fish were injected at dose of $1.0\times10^7$ CFU/fish; B: fish were injected at dose of $1.0\times10^6$ CFU/fish.

### 3.7. $LD_{50}$ of flounder isolate of *E. tarda* or *A. hydrophila* to 10 g catfish or tilapia

To accurately compare the virulence between *E. tarda* isolate and *A. hydrophila* isolate to channel catfish or Nile tilapia, fish at similar size (10 g) were used in this study. The $LD_{50}$ values of the Southern flounder isolate of *E. tarda* or *A. hydrophila* to catfish or tilapia are summarized in Table 2. When healthy catfish were exposed to the Southern flounder isolates by intraperitoneal injection, the $LD_{50}$ values of *E. tarda* or *A. hydrophila* to 10 g catfish were $6.1\times10^4$ and $1.4\times10^7$ CFU/fish, respectively (Table 2). Based on $LD_{50}$ values, the Southern flounder isolate of *E. tarda* was 230-fold more virulent to 10 g catfish than the Southern flounder isolate

of *A. hydrophila*. When healthy tilapia were exposed to the Southern flounder isolates by intraperitoneal injection, the $LD_{50}$ values of *E. tarda* or *A. hydrophila* to 10 g tilapia were $1.1\times10^7$ and $5.6\times10^7$ CFU/fish, respectively (Table 2). Based on $LD_{50}$ values, the Southern flounder isolate of *E. tarda* was 5-fold more virulent to 10 g tilapia than the Southern flounder isolate of *A. hydrophila*. When the same bacterial isolate was used, catfish were 180- and 4-fold more susceptible to infection by the Southern flounder isolate of *E. tarda* and *A. hydrophila* infection, respectively.

**Table 2**

$LD_{50}$ values of Southern flounder isolates of *E. tarda* or *A. hydrophila* to 10 g channel catfish or Nile tilapia by intracoelomic injection.

| Isolate name | | $LD_{50}$[a] (95% *CI*)[b] | Slope (SE) | $\chi^2$ |
|---|---|---|---|---|
| To 10 g catfish | *E. tarda* | $6.1\times10^4$ ($2.8\times10^4$-$1.3\times10^5$)$^{A}$ | 1.01 (0.12) | 1.04 |
| | *A. hydrophila* | $1.4\times10^7$ ($2.4\times10^6$-$3.4\times10^7$)$^{B}$ | 2.53 (0.27) | 4.26 |
| To 10 g tilapia | *E. tarda* | $1.1\times10^7$ ($2.9\times10^6$-$8.4\times10^7$)$^{B,C}$ | 0.59 (0.11) | 0.42 |
| | *A. hydrophila* | $5.6\times10^7$ ($4.6\times10^7$-$7.5\times10^7$)$^{C}$ | 3.56 (0.65) | 0.95 |

[a]$LD_{50}$ values are in units of colony forming unit per fish. [b]95% *CI*, virulence was considered significantly different when 95% *CI* failed to overlap. $^{A,B,C}$: same letters indicate that the virulence was not significantly different from each other because 95% *CI* overlapped with each other. Different letters indicate that the virulence was significantly different from each other because 95% *CI* failed to overlap.

## 4. Discussion

In the summer of 2012, mass mortality of Southern flounder was observed in North Carolina, USA, with infected fish showing loss of pigmentation, exophthalmia, opacity of the eyes, swelling of the abdominal surface, and rectal hernia. Based on symptoms, Edwardsiellosis was suspected since olive flounder infected with *E. tarda* also showed loss of pigmentation and rectal hernia[11]. From the diseased Southern flounder, 25 Gram-negative bacterial isolates were cultured. Based on API 20NE and FAME analysis, the 25 isolates were found to share homologies with either *E. tarda* or *A. hydrophila*. Based on sequencing results of partial 16S rRNA gene, 15 isolates shared 100% identities (*E* value=0) with the 16S rRNA sequence of *E. tarda* strain TX1 (GenBank EF467363), whereas the rest 10 isolates shared 100% identities (*E* value=0) with the 16S rRNA sequence of *A. hydrophila* strain An4 (GenBank FJ386959).

Virulence of Southern flounder isolates of *E. tarda* or *A. hydrophila* to different sizes of Nile tilapia revealed that larger tilapia were more resistant to infection than smaller ones. When mortality data were divided by fish weight, smaller fish had significantly higher mortality per gram of fish. Effect of fish sizes or weights on their susceptibility to bacterial infection has been reported previously by Pasnik *et al*[12]. For example, when two sizes of channel catfish (15 g fingerling and 55 g juvenile) were exposed to *Streptococcus ictaluri* by intraperitoneal injection, 14% mortality per gram of fish was observed in fingerlings whereas only 6% mortality per gram of fish was observed in juveniles[12]. Taken together, these results suggest that the size of fish might have an effect on their susceptibility to pathogens.

Mortality data revealed that the *E. tarda* isolate cultured from Southern flounder was more virulent than the *A. hydrophila* isolate. Based on $LD_{50}$ values, the Southern flounder isolate of *E. tarda* was 5- and 230- fold more virulent to 10 g tilapia and catfish, respectively. These results suggested that *E. tarda* could be the primary pathogen that caused the disease outbreak in the Southern flounder in the summer of 2012. Based on $LD_{50}$ values, compared to Nile tilapia, channel catfish were 180- and 4-fold more susceptible to infection by the Southern flounder isolate of *E. tarda* and *A. hydrophila* infection, respectively. Previous research study of Pridgeon *et al*. has also revealed that channel catfish were more susceptible to infection by *E. tarda* or *A. hydrophila* compared to Nile tilapia[13]. For example, when similar dose and similar size of fish were used in virulence studies, 100% channel catfish were killed by nine isolates of *E. tarda*, whereas only an average of 40% of tilapia were killed[13]. Taken together, these results suggest that Nile tilapia in general are more resistant to *E. tarda* and *A. hydrophila* compared to channel catfish.

In summary, 25 Gram-negative bacteria isolates were recovered from tissues of diseased Southern flounder. Based on API 20NE and FAME analysis, the 25 isolates were found to share homologies with either *E. tarda* or *A. hydrophila*. Based on sequencing results of partial 16S rRNA gene, 15 isolates shared 100% identities with the 16S rRNA sequence of *E. tarda* strain TX1 (GenBank EF467363), whereas the remaining 10 isolates shared 100% identities with the 16S rRNA sequence of *A. hydrophila* strain An4 (GenBank FJ386959). When healthy fish were exposed to flounder isolate by intraperitoneal injection, the $LD_{50}$ values of flounder isolate *E. tarda* to 10 g channel catfish or Nile tilapia were $6.1\times10^4$ and $1.1\times10^7$ CFU/fish, respectively, whereas that of *A. hydrophila* to channel catfish and Nile tilapia were $1.4\times10^7$ and $5.6\times10^7$ CFU/fish, respectively. Based on $LD_{50}$ values, the Southern flounder isolate of *E. tarda* was 5- and 230-fold more virulent to 10 g tilapia and catfish, respectively. These results suggested that *E. tarda* could be the primary pathogen that caused the disease outbreak in the Southern flounder in the summer of 2012.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgements

We thank Drs. Dunhua Zhang (USDA-ARS) and Victor Panangala (USDA collaborator) for critical reviews of the manuscript. We thank Dr. Brian Scheffler and Fanny Liu (USDA-ARS-Catfish Genetics Research Unit) for their excellent sequencing work. We thank Beth Peterman and Julio Garcia (USDA-ARS), Kent Passingham and Adriane Gill (North

Carolina State University) for their technical support. This study was supported by the USDA/ARS CRIS project #6420-32000-024-00D. The use of trade, firm, or corporate names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the United States Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

## Comments

### *Background*

Gram-negative bacteria *A. hydrophila* and *E. tarda* are widely distributed in natural environment and can cause diseases in many fish species including human. In fish, a lot of isolates of them were found to be respectively virulent to marine or freshwater fish species. While, for some isolates, whether they can cause diseases in both marine and fresh water species is unknown.

### *Research frontiers*

The present research work demonstrated that *E. tarda* could be the primary pathogen that caused the disease outbreak in the Southern flounder in the summer of 2012. This research results suggest that the size of fish might have an effect on their susceptibility to pathogens.

### *Related reports*

In other research, it was also found that channel catfish were more susceptible to infection by *E. tarda* or *A. hydrophila* compared to Nile tilapia. The virulence of *E. tarda* or *A. hydrophila* isolated from freshwater were also detected in channel catfish and Nile tilapia.

### *Innovations and breakthroughs*

The present research work demonstrated that *E. tarda* could be the primary pathogen that caused the disease outbreak in the Southern flounder in the summer of 2012 and these bacteria isolated from diseased Southern flounder are virulent to channel catfish and Nile tilapia.

### *Applications*

This scientific study will give a guide for controlling disease outbreak in the Southern flounder in North Carolina, USA since *E. tarda* is regarded as the primary pathogen that caused the disease outbreak. This study also demonstrated bacteria isolated from marine species can also cause disease in freshwater species.

### *Peer review*

This is a valuable research work in which authors have demonstrated *E. tarda* could be the primary pathogen that caused the disease outbreak in the Southern flounder in the summer of 2012 and these bacteria isolated from diseased Southern flounder are virulent to channel catfish and Nile tilapia. This research results suggest that the size of fish might have an effect on their susceptibility to pathogens.

## References

[1] Park CI, Choi SY, Kim WS, Oh MJ, Kim EH, Kim HY, et al. Characterization of the unusual non-thiosulfate-reducing *Edwardsiella tarda* isolated from eel (*Anguilla japonica*) farms. *Aquaculture* 2012; **356-357**: 415-419.

[2] Alcaide E, Herraiz S, Esteve C. Occurrence of *Edwardsiella tarda* in wild European eels *Anguilla anguilla* from Mediterranean Spain. *Dis Aquat Organ* 2006; **73**: 77-81.

[3] Sun Y, Li N, Song J, Li X, Shen H, Wang F, et al. Isolation and identification of bacterial pathogen *Aeromonas hydrophila* and development of the inactivated vaccine in Japanese flounder *Paralichthys olivaceus* diseased with ascites. *Fish Sci (Dalian)* 2009; **28**: 613-617.

[4] Rajpakshe AD, Prasad KP, Mukherjee SC, Kumar K, Brahmachari RK, Meena CT, et al. *In vitro* sensitivity of three bacterial pathogens of koi carp (*Cyprinus carpio* L.) to certain antibiotics. *J Agric Sci Technol* 2012; **2**: 93-98.

[5] Tekedar HC, Karsi A, Williams ML, Vamenta S, Banes MM, Duke M, et al. Complete genome sequence of channel catfish gastrointestinal septicemia isolate *Edwardsiella tarda* C07-087. *Genome Announc* 2013; **1**(6): e00959-13. doi:10.1128/genomeA.00959-13.

[6] Leung KY, Siame BA, Tenkink BJ, Noort RJ, Mok YK. *Edwardsiella tarda*-virulence mechanisms of an emerging gastroenteritis pathogen. *Microbes Infect* 2012; **14**: 26-34.

[7] Zhou Z, Guo D. Catheter-related bacteremia caused by *Aeromonas hydrophila* in a hemodialysis patient. *Infect Control Hosp Epidemiol* 2013; **34**: 765-766.

[8] Pridgeon JW, Klesius PH. Major bacterial diseases in aquaculture and their vaccine development. *CAB Rev Perspect Agric Vet Sci Nutr Nat Res* 2012; **7**: 1-16.

[9] Notredame C, Higgins DG, Heringa J. T-Coffee: a novel method for fast and accurate multiple sequence alignment. *J Mol Biol* 2000; **302**: 205-217.

[10] Di Tommaso P, Moretti S, Xenarios I, Orobitg M, Montanyola A, Chang JM, et al. T-Coffee: a web server for the multiple sequence alignment of protein and RNA sequences using structural information and homology extension. *Nucleic Acids Res* 2011; **39**(Web Server issue): W13-W17.

[11] Park SB, Aoki T, Jung TS. Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish. *Vet Res* 2012; **43**: 67.

[12] Pasnik DJ, Evans JJ, Klesius PH, Shoemaker CA, Yeh HY. Pathogenicity of *Streptococcus ictaluri* to channel catfish. *J Aquat Anim Health* 2009; **21**: 184-188.

[13] Pridgeon JW, Klesius PH, Song L, Zhang D, Kojima K, Mobley JA. Identification, virulence, and mass spectrometry of toxic ECP fractions of West Alabama isolates of *Aeromonas hydrophila* obtained from a 2010 disease outbreak. *Vet Microbiol* 2013; **164**: 336-343.