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

Julia W. Pridgeon1, Phillip H. Klesius1, Gregory A. Lewbart2, Harry V. Daniels3, Megan Jacob2

1Aquatic Animal Health Research Unit, USDA-ARS, 990 Wire Road, Auburn, AL 36832, United States of America
2College of Veterinary Medicine, North Carolina State University, 1060 William Moore Drive, Raleigh, NC 27607, United States of America
3Department of Applied Ecology, North Carolina State University, Raleigh, NC 27695, United States of America

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 LD50 values of flounder isolate E. tarda to channel catfish or Nile tilapia [10±2 g] were 6.1±107 and 1.1±108 CFU/fish, respectively, whereas that of flounder isolate A. hydrophila to channel catfish and Nile tilapia were 1.4±106 and 5.6±106 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
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. Two sterile loops were used to inoculate the tissue samples onto tryptic soy agar (TSA) plates. After overnight growth at 28°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 (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 mmx100 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 1.00. 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 GGT TGA TCG TCT AGG–3′ and universal 16S reverse primer 5′–AAG GAG GTG TCC TAA GCC–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 H2O, 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 1.0×10⁸ CFU/fish. For 10 g tilapia, two doses (1.0×10⁷ and 1.0×10⁸ 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 LD₅₀ 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 1×10⁷ to 1×10⁸ 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 LD₅₀ values. Virulence of *E. tarda* and *A. hydrophila* was considered significantly different when the 95% confidence intervals of LD₅₀ values failed to overlap (P≤0.05).

### 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.

#### 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).

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). These results were analyzed using 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 1.0x10^4 CFU/fish, all eight bacterial isolates killed 100% Nile tilapia at size of 10 g (Table 1). However, at dose of about 1.0x10^5 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 of 1.0x10^5 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).

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.

Figure 3. BlastN search results of the partial 16S rRNA sequence of the Southern flounder isolate of E. tarda.
Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Injection dose (CFU/fish)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>E. tarda isolate #1</td>
<td>1.0x10^7</td>
<td>100%</td>
</tr>
<tr>
<td>E. tarda isolate #5</td>
<td>1.0x10^7</td>
<td>100%</td>
</tr>
<tr>
<td>E. tarda isolate #7</td>
<td>1.0x10^7</td>
<td>100%</td>
</tr>
<tr>
<td>E. tarda isolate #10</td>
<td>1.0x10^7</td>
<td>100%</td>
</tr>
<tr>
<td>E. tarda isolate #12</td>
<td>1.0x10^7</td>
<td>100%</td>
</tr>
<tr>
<td>A. hydrophila isolate #19</td>
<td>1.0x10^7</td>
<td>100%</td>
</tr>
<tr>
<td>A. hydrophila isolate #20</td>
<td>1.0x10^7</td>
<td>100%</td>
</tr>
<tr>
<td>A. hydrophila isolate #23</td>
<td>1.0x10^7</td>
<td>100%</td>
</tr>
<tr>
<td>E. tarda isolate #1</td>
<td>1.0x10^7</td>
<td>80%</td>
</tr>
<tr>
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</tr>
<tr>
<td>E. tarda isolate #7</td>
<td>1.0x10^7</td>
<td>80%</td>
</tr>
<tr>
<td>E. tarda isolate #10</td>
<td>1.0x10^7</td>
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<tr>
<td>E. tarda isolate #12</td>
<td>1.0x10^7</td>
<td>70%</td>
</tr>
<tr>
<td>A. hydrophila isolate #19</td>
<td>1.0x10^7</td>
<td>13.8%</td>
</tr>
<tr>
<td>A. hydrophila isolate #20</td>
<td>1.0x10^7</td>
<td>10%</td>
</tr>
<tr>
<td>A. hydrophila isolate #23</td>
<td>1.0x10^7</td>
<td>8.8%</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>8.8%</td>
</tr>
</tbody>
</table>

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 LD₅₀ 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 LD₅₀ values of E. tarda or A. hydrophila to 10 g catfish were 6.1x10⁷ and 1.4x10⁹ CFU/fish, respectively (Table 2). Based on LD₅₀ 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 LD₉₀ values of *E. tarda* or *A. hydrophila* to 10 g tilapia were 1.1×10⁶ and 5.6×10⁷ CFU/fish, respectively (Table 2). Based on LD₉₀ 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**

<table>
<thead>
<tr>
<th>Isolate name</th>
<th>LD₉₀ (95% CI)</th>
<th>Slope (SE)</th>
<th>χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>To 10 g catfish</td>
<td><em>E. tarda</em></td>
<td>6.1×10⁵ (2.8×10⁵–1.3×10⁶)</td>
<td>1.01 (0.12)</td>
</tr>
<tr>
<td><em>A. hydrophila</em></td>
<td>1.4×10⁶ (2.4×10⁵–3.4×10⁶)</td>
<td>2.53 (0.27)</td>
<td>4.26</td>
</tr>
<tr>
<td>To 10 g tilapia</td>
<td><em>E. tarda</em></td>
<td>1.1×10⁶ (2.9×10⁵–8.4×10⁵)</td>
<td>0.99 (0.11)</td>
</tr>
<tr>
<td><em>A. hydrophila</em></td>
<td>5.6×10⁶ (4.6×10⁵–7.5×10⁶)</td>
<td>3.56 (0.65)</td>
<td>0.95</td>
</tr>
</tbody>
</table>

LD₉₀ values are in units of colony forming unit per fish. 95% CI, virulence was considered significantly different when 95% CI failed to overlap. *a*: 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 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 LD₉₀ values of flounder isolate *E. tarda* to 10 g channel catfish or Nile tilapia were 6.1×10⁵ and 1.1×10⁷ CFU/fish, respectively, whereas that of *A. hydrophila* to channel catfish and Nile tilapia were 1.4×10⁷ and 5.6×10⁷ CFU/fish, respectively. Based on LD₉₀ 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 LD₉₀ 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 LD₉₀ values of flounder isolate *E. tarda* to 10 g channel catfish or Nile tilapia were 6.1×10⁵ and 1.1×10⁷ CFU/fish, respectively, whereas that of *A. hydrophila* to channel catfish and Nile tilapia were 1.4×10⁷ and 5.6×10⁷ CFU/fish, respectively. Based on LD₉₀ 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
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**


