ACTA SCIENTIFIC MICROBIOLOGY (ISSN: 2581-3226)

Volume 2 Issue 1 January 2019

Research Article

# Occurrence of *Edwardsiella tarda* in Nile Tilapia *Oreochromis niloticus* from Brazilian Aquaculture *Edwardsiella tarda* in Nile Tilapia

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**Received:** November 20, 2018; **Published:** December 14, 2018

## Abstract

*Edwardsiella tarda* is a gram-negative bacterium that causes high economic loss in aquaculture systems due its morbidity and mortality in different species of fish, such as Nile tilapia *Oreochromis niloticus*. The main problem is that this microorganism can also infect humans. Due to the importance of this bacteriosis, the aim of this study was to detect the presence of *E. tarda* by PCR and describe lesions in these positive samples by necropsy and histopathology in Nile tilapia *Oreochromis niloticus* of cage fish farms localized in hydroelectrical reservoirs of São Paulo state, Brazil. For this, 360 *O. niloticus* were sampled from 6 fish farms in two moments: November 2014 (N = 180) and in March 2015 (180). All fish were euthanized and submitted to necropsy and sampling the organs to histopathology and PCR. The fishes with edwardsiellosis presented hemorrhage, ocular edema, opacity of the cornea, splenomegaly, and ulcer of the skin. In histopathology were seen bacterial colonies, necrosis, granulomes, hemorrhages, inflammatory cells, and melano-macrophages. After sequencing the positive samples, our data presented 96% of identity with *Edwardsiella tarda* strain FL95-01 (genbank: CP011359) isolated in USA from catfish. Outbreaks and surveillance of *E. tarda* in Brazil is poorly reported, but present in many states of Brazil, which could cause disease in humans from Brazil and other countries, once *O. niloticus* is exported to many countries and the habits of intake raw fish by humans is high.

**Keywords:** Edwardsiellosis; Zoonosis; PCR; Histopathology

## Introduction

In the last years, aquaculture has increased worldwide, this intensification has been accompanied with high stock densities and other stress factors which makes fish more vulnerable to adverse impacts of disease and environmental conditions. It is known that representatives of many bacterial taxa have been associated with fish diseases and that this has become a great concern to the aquaculture [1]. However, one of the most significant bacterial pathogens to farmed fish is *Edwardsiella tarda*, that has been reported to cause disease in many economically important fish species worldwide, as in the USA [2,3], China [4], India [5], Japan [6]. Spain [7] and Brazil [8,9].

*E. tarda* is a Gram-negative, facultatively anaerobic, motile, peritrichously flagellated, rod-shaped bacterium of the family Enterobacteriaceae and phylum Proteobacteria [10]. Infection by this bacterium often leads to the development of a systematic disease called edwardsiellosis characterized by symptoms of ascites, her-

nia, exophthalmia and severe lesions of internal organs [11]. This microorganism is an intracellular pathogen and is capable of infecting various types of cells [12-14] and in a broad range of hosts like fish, amphibians, reptiles, birds and mammals, including humans [15,16]. The mode of transmission of disease is by means of exposure to aquatic environments and in case of humans and animals of ingestion of contaminated fish [17].

Edwardsiellosis in fish usually occur under imbalanced environmental conditions, such as high water temperature, poor water quality, and high organic content [18]. Fish infected with *E. tarda* show abnormal swimming behavior, including spiral movement and floating near the water surface. The fish too reveal gross lesions on the skin, pale gills, tumefaction of the eye, excessive mucus secretion, scale erosion and ulcers in a few cases [3]. Fish seem to be the most susceptible animal to *E. tarda* compared with other animals, with a mortality rate up to 90-95% in adult turbot [19]. Since this bacterium is an intracellular pathogen, most antibiotics are not very effective for this disease [11], besides most strains are resistant to drugs [16]. Due these factors, the edwardsiellosis have represented great loss for the fish production and a serious public health problem. Thus, the aim of this study was to detect the presence of *E. tarda* by PCR and describe lesions in these samples by necropsy and histopathology in Nile tilapia *Oreochromis niloticus* of cage fish farms localized in hydroelectrical reservoirs of São Paulo state, Brazil.

## Methods

### Ethics statement

This study was carried out in strict accordance with the recommendations in the following laws: Law 11794/2008 and Decree 6899/2009. The protocol was approved by the Ethics Committee on Animal Use of the São Paulo State University (UNESP) (Protocol Number: 724-CEUA). The owners of fish farms used in this study consent the use of their fish for detecting pathogens and other analysis, which is not present in this paper.

### Sampling

*Oreochromis niloticus* specimens were randomly sampled at six Brazilian fish farms in November 2014 (N = 180) and in March 2015 (180) (Table 1). Each fish-farm and sampling was named as A(1): fish farm 1 (first sampling), A(2): fish farm 1(second sampling), B(1): fish farm 2 (first sampling), B(2): fish farm 2 (second sampling), C(1): fish farm 3 (first sampling), C(2): fish farm 3 (second sampling), D(1): fish farm 4 (first sampling), D(2): fish farm 4 (second sampling), E(1): fish farm 5 (first sampling), E(2): fish farm 5 (second sampling), F(1): fish farm 6 (first sampling), and F(2): fish farm 6 (second sampling).

| Fish Farm | N | Weight[1] (g) ($\bar{x}$) | Weight[2] (g) ($\bar{x}$) | Size[1] (cm)($\bar{x}$) | Size[2] (cm)($\bar{x}$) |
|---|---|---|---|---|---|
| A | 30 | 518.67 | 441.10 | 19.53 | 21.64 |
| B | 30 | 434.76 | 518.99 | 22.33 | 22.00 |
| C | 30 | 286.55 | 537.56 | 19.49 | 22.02 |
| D | 30 | 427.47 | 430.86 | 21.42 | 20.73 |
| E | 30 | 234.08 | 396.82 | 18.43 | 22.77 |
| F | 30 | 304.17 | 424.77 | 19.71 | 21.49 |
| Total | 180 | 367.61 | 458.35 | 20.15 | 21.77 |

**Table 1**: Number, weight (g), and size (cm) of *Oreochromis niloticus* sampled at the six fish farms in the first and second sampling events.

[1]First sampling; [2]Second sampling.

Necropsy was performed according to Noga [20]. The organs sampled were the brain, gall bladder, gill, gut, heart, kidney, liver, muscle, spleen, and stomach for histopathology and molecular analysis. According to Noga [20], a 1-cm$^3$ portion of each tissue was fixed in 10% neutral buffered formalin followed by processing using standard histological techniques and embedded in paraffin. Hematoxylin and eosin were used for staining.

### DNA extraction, PCR (polymerase chain reaction), sequencing, and phylogeny test

The organ tissues collected from each fish were pooled and 20 mg of each sample was used for molecular analysis. The DNA extraction was performed using the Wizard® SV Genomic DNA Purification System kit (Promega Corporation®) according to the manufacturer's instructions. The DNA was eluted in elution buffer (nuclease-free water) and kept at -20°C until use. Purity and quantification of extracted DNA was measured using a 260/280 absorbance rate in a Nanodrop 2000c (Thermo Fisher Scientific®). Only DNA with a ratio of >1.7 (260/280 rate) was used in this study.

Because we observed some lesions compatible with bacterial diseases in the necropsy and histology, we performed a PCR (polymerase chain reaction) that recognize fimbrial subunit gene of *Edwardsiella tarda* according to Sakai., *et al* [21]. For this purpose, the reaction mixture consisted of 10 µL de Gotaq qPCR Mastermix 2X (Promega), 10 pmol of each primer (EtFimDF and EtFimDR),

3 µL de DNA, and water nucleasse free to adjust to 20 µL. The reaction consisted of an initial denaturation step of 94°C for 2 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, finishing with terminal extension at 72°C for 7 min and a hold at 22°C. The 445 bp amplicons were purified with an Ilustra Microspin™ S-400 HR Columns Kit (GE Healthcare®) according to the manufacturer's instructions for identification by Sanger sequencing. For this purpose, the purified amplicon was sequenced in both directions using BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems) on an Applied Biosystems capillary 3500 Genetic Analyzer. The quality of the electropherograms was assessed in Sequencing Analysis version 5.4 (Applied Biosystems). After this step, sequences were identified by similarity using BLAST (Basic Local Alignment Search Tool) algorithm.

The nucleotide sequence of approximately 400 bp was used to query the GenBank library to arrive at the closest type strain and thus attain a species affiliation and possible identification to that level. To compare the sequences from different strain found in Genbank library, the nucleotide sequences were aligned with ClustalW from MEGA software, version 7, and dendrograms were created by using the neighbor-joining method based on a model by Jukes and Cantor.

### Statistical analysis

The prevalence of *Edwardsiella tarda* detected by PCR was calculated for each fish farm sampled in the two moments (November 2014 and March 2015). The occurrence of lesions observed (necropsy and histopathology) and positive PCR results were also analyzed. All statistical analyses were performed in Statistica v. 10 [22] and visualized in GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

## Results

### Lesions

Major of fish analyzed during this study presented many lesions, as hepatomegaly, liquefaction of the kidney, and melanisation of the skin. Although, those which was positive by molecular techniques for *E. tarda* had as most prevalent hemorrhage and splenomegaly (Table 2). Other signs were also observed as ulcer of the skin, opacity of the cornea, and ocular edema. In the histopathology, we visualized melano-macrophages in higher quantity in the spleen and kidney, inflammatory process, coagulative necrosis, vacuolar degeneration, and bacteria colonies with the most occurrence (Table 3). Although, other alterations were present in lower quantity: congestion, hemorrhage, calcification necrosis, granulomes in the spleen and kidney (Figure 1), and eosinophils cells.

| Lesions | A | B | C | F |
|---|---|---|---|---|
| Haemorrhage | 100.00 (8/8) | 33.33 (1/3) | 66.66 (2/3) | 9.09 (1/11) |
| Ocular oedema | 12.50 (1/8) | 0.00 (0/3) | 0.00 (0/8) | 0.00 (0/11) |
| Opacity of the cornea | 12.50 (1/8) | 0.00 (0/3) | 0.00 (0/8) | 0.00 (0/11) |
| Splenomegaly | 100.00 (8/8) | 0.00 (0/3) | 100.00 (3/3) | 63.63 (7/11) |
| Ulcer of the skin | 25.00 (2/8) | 0.00 (0/3) | 33.33 (1/3) | 0.00 (0/11) |

**Table 2.** Prevalence of macroscopic lesions observed in *Oreochromis niloticus* positive for *Edwardsiella tarda* by PCR.

| Lesions | A | B | C | F |
|---|---|---|---|---|
| Bacteria colonies | 87.50 (7/8) | 66.66 (2/3) | 0.00 (0/3) | 100.00 (11/11) |
| Calcification necrosis | 25.00 (2/8) | 33.33 (1/3) | 0.00 (0/3) | 9.09 (1/11) |
| Coagulative necrosis | 100.00 (8/8) | 100.00 (3/3) | 66.66 (2/3) | 81.81 (9/11) |
| Congestion | 0.00 (0/8) | 0.00 (0/3) | 33.33 (1/3) | 18.18 (2/11) |
| Eosinophils cells | 12.5 (1/8) | 33.33 (1/3) | 0.00 (0/3) | 18.18 (2/11) |
| Granulomas | 0.00 (0/8) | 33.33 (1/3) | 66.66 (2/3) | 36.36 (4/11) |
| Haemorrhage | 50.00 (4/8) | 66.66 (2/3) | 33.33 (1/3) | 100.00 (11/11) |
| Inflammatory process | 100.00 (8/8) | 100.00 (3/3) | 100.00 (3/3) | 63.63 (7/11) |
| Melano-macrophages | 100.00 (8/8) | 100.00 (3/3) | 33.33 (1/3) | 100.00 (11/11) |
| Vacuolar degeneration | 100.00 (8/8) | 100.00 (3/3) | 100.00 (3/3) | 81.81 (9/11) |

**Table 3.** Prevalence of microscopic lesions observed in *Oreochromis niloticus* positive for *Edwardsiella tarda* by PCR.

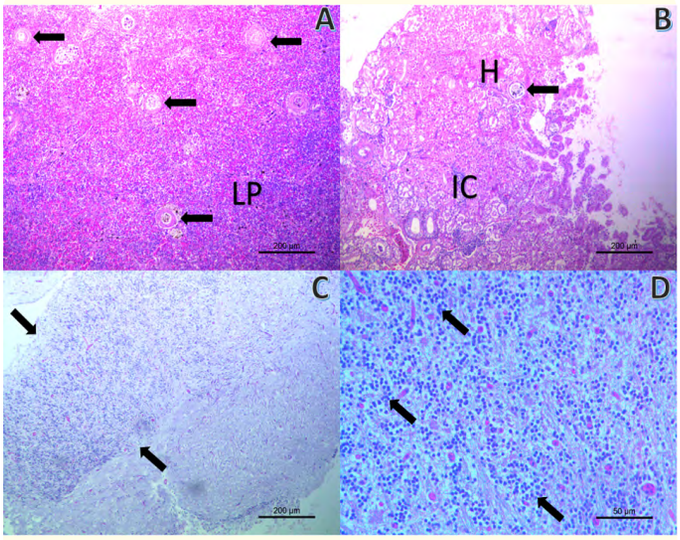


**Figure 1:** Lesions observed in histopathology. A. Granulomas (arrow) and lymphocyte proliferation (LP) in the spleen. B. Granulomas (arrow), haemorrhage (H), and inflammatory cells (IC) in the kidney. C and D. Inflammatory cells (arrow) in the brain. Haematoxylin and Eosin.

CP011359). After this confirmation, we observed that in the first sampling, only fish farms 1 (A), 2 (B), and 3 (C) were positive for this bacterium (Figure 2). Although, in the second sampling, only fish farm 6(F) had fishes with edwardsiellosis.

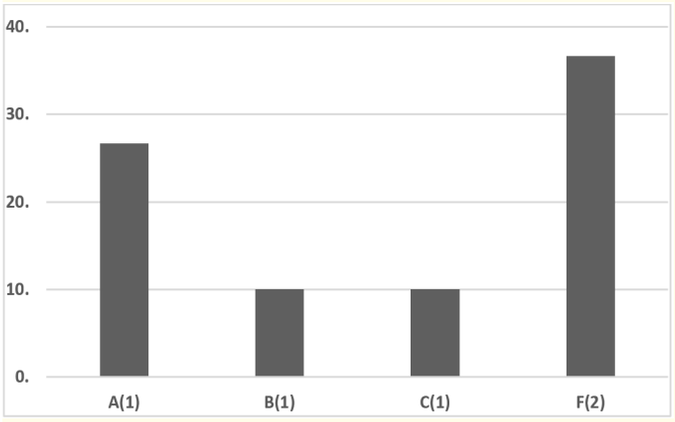


**Figure 2:** Prevalence (%) of *Edwardsiella tarda* by PCR in Oreochromis niloticus sampled from fish farms positives in São Paulo State, Brazil.

## Molecular results

The amplification of fymbrial subunit gene amplified a product of 445 bp, which presented in the sequencing analysis 96% (384/400) of identity with *E. tarda* strain FL95-01 (genbank:

In the blastn search (GenBank) were found nine sequences with similarity until 72% and score >200 with the sequence of this study, being six *E. tarda* isolates (Figure 3). These isolates were from 6 fish species of 5 countries spread in 2 continents.


**Figure 3:** Maximum Parsimony model with 1000 bootstrap replicates. The sequence in red was found in this study. The tree shows respectively for each isolate: the country and the host from which they were isolated, the identity with the sequence of this study, the size of complete genome in kbp, and accession number from GenBank.

## Discussion

*Edwardsiella* is a Gram-negative bacterium that could be found in the intestine of healthy fish [15,23], but Mohanty and Sahoo [15] and Park., *et al*. [16] reported that them could cause disease in fish, amphibians, reptiles, birds and mammals, including humans throughout the world. *E. tarda* can cause diseases in many species of fish, including *Oreochromis niloticus* [15,16,24-26], which was found in this study. An important point is that according to Hawke., *et al*. [27], this specie of *Edwardsiella* is the only specie responsible to zoonosis, highlighting the importance of diagnosis this disease and alerts the people who works directly with these fishes infected. The main way of infection is by intake of raw seafood infected, which could lead to watery to bloody diarrhea prolonged or intermittent, anorexia, and vomiting specially in immunocompromised or immunodepressed patients, older adults and children [28-32]. This is an important point due to the increase of intake of raw tilapia.

*Edwardsiella tarda* in *O. niloticus* was reported in Brazil at Minas Gerais state with prevalence from 30.5-60%, being the most cases in the summer (52%) and autumn (60%) [24]. Other states detected this bacteriosis, such as Mato Grosso do Sul [33], Rio Grande do Sul [34], and Bahia [25]. In this study we observed prevalence from 10 to 36.67% in *O. niloticus* from São Paulo state. The presence of this bacteria in fish could vary due to stress of the fish and water quality [20]. Although these parameters are not showed in this paper, we observed during sampling high density of the fishes as poor quality of the water, which can contribute for this disease. Another important point is that some fish farms and moments not present *E. tarda*, probably due to good practices of production or water quality.

*Edwardsiellosis* is the name of the disease caused by Edwardsiella bacteria. Infection can lead to the development of a systematic disease, which could be detected in animals who presents ascites, hernia, exophtalmia, brain and severe lesions of the internal organs [11]. In a review made by Xu and Zhang [11] of edwardsiellosis in fish, the authors saw that the most common lesions that were reported are ascites, exophthalmia, liver granulomas, swollen in internal organs, hemorrhages and red-head disease. According to Rey., *et al.* [35] a recent outbreak of edwardsiellosis in tilapia hybrids presented ocular lesions (exophthalmia, corneal opacity, and eyes losing), abdominal distension, nodules in the caudal fin, and hemorrhagic ulceration of the urogenital orifice. Could be present also enlargement of the liver, spleen, and kidney exhibiting whitish nodules. In this study we detected the same lesions as described by others researches in the eyes (i.e., ocular edema and opacity of the cornea), hemorrhage, ulcer, and splenomegaly (Table 2), showing that the infection of these fishes could be caused by *Edwardsiella tarda*.

Microscopically, the type of inflammatory response induced by *E. tarda* may differ between fish species [36]. This may be due to the fish species itself, the phase of infection, the virulence factors produced by different strains of this microorganism, as well as the fish condition to mount a weak or strong inflammatory response [37]. Miyazaki and Kaige [36] described that the infection in tilapia and red sea bream is characterized by granulomatous inflammation preceded by macrophage infiltration and a reduced number of bacteria inside the granulomas. As mentioned by these authors, we also observed an increase of macrophages and inflammatory response, and granuloma formation (Table 3). Although, in this study it was seen another process, such as coagulative necrosis, vacuolar degeneration, bacteria colonies in the interstice, congestion, hemorrhage, calcification necrosis, and eosinophils cells. Iregui., *et al*. [38] observed necrosis in tilapia with edwardsiellosis, but only in the nervous system, differing from this study. The degeneration was an alteration also visualized in the intestine by Garcia., *et al.* [37], results similar from this research.

Our bioinformatics study (until ID 72%) revealed that our sequence was similar to nine *Edwardsiella* sp. isolates from 6 fish species of 5 countries, being six *E. tarda* isolates from 4 fish species of 4 countries spread in 2 continents (Figure 3) could evidences the global distribution of this pathogen. Of all sequences, the *E. tarda* KG8401 was the one who did not show the genome completely sequenced in NCBI [6]. The complete genome of sequences varies from 3,620,701(strain FL95-01) to 4,200,387 (strain 080813). The sequence closest of sequence this study was to *E. tarda* FL95-01 (96% ID) isolated in the USA from catfish, your genome consists of one circular chromosome with 3,620,701 bp, 57.3% GC content, 3,258 genes encoding 3,091 proteins and 101 tRNAs [39]. The complete genome of *E. tarda* isolate FL95-01 shares average nucleotide identities (ANI) of 83.4% with the isolate C07-087 isolated also from catfish in the USA. *E. tarda* C07-087 does not carry any plasmids, however, 31,387 bp of the plasmid (44,194-bp) of the *E. tarda* FL6-60 is integrated into the *E. tarda* strain C07-087 chromosome [40]. The *E. piscicida*-like sp. LADL05-105 (72% ID)

was the closest of the sequence from this study found in Tilapia but this from USA. This strain consists of one circular chromosome with 4,142,037 bp the biggest of our bioinformatics study. The complete genome of LADL05-105 shares an ANI of 99.84% with the strain 080813, which was recently proposed as the type strain for *Edwardsiella anguillarum*. The isolate also shares 99.59% ANI with *E. piscicida*-like sp. isolate EA181011, 94.39% with *E. tarda* isolate C07-087 and 84.01% with *E. tarda* isolate FL95-01 [41].

## Conclusion

In conclusion, outbreaks and surveillance of *E. tarda* in Brazil is poorly reported, but present in many states of Brazil, which could cause disease in humans from Brazil and other countries, once *O. niloticus* is exported to many countries and the habits of intake raw fish by humans is high. For this, it is important to diagnosis and perform surveillance programs to this bacteriosis avoiding this contaminated fish for human consumption.

## Acknowledgements

The authors thank to FAPESP for the financial support (2013/50504-5) and postdoctoral fellowships (2014/15859-0 to C. J. Francisco, 2015/13025-7 to M.F Falcone- Dias, and 2014/13718-0 to M. V. Rodrigues). The authors also thanks to CNPq for the doctor the fellowship to R. J. da Silva of CNPq (307808/2014-9) and CNPq-PROTAX (440496/2015-2)/FAPESP 2016/50377-1.

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**Volume 2 Issue 1 January 2019**