Research Article

Aeromonas hydrophila in Nile tilapia (*Oreochromis niloticus*) from Brazilian aquaculture: a public health problem

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Abstract

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Aeromonas hydrophila is a Gram-negative bacterium present in the water, which can cause disease in animals, such as fish, frog, and mammals, including humans. In fish, Aeromonosis occurs when it is immunosuppressed due to the stress of handling, water quality, parasitism or population density. Due the importance of this disease in fish and humans, this study aimed to detect this bacterium by PCR in Nile tilapia (*Oreochromis niloticus*) of cage fish farms localized in hydro-electrical reservoirs of São Paulo state, Brazil and describe the lesions found in positive fish by necropsy and histopathology. Around 360 samples of *Oreochromis niloticus* specimens were randomly sampled at six Brazilian fish farms in November 2014 (n = 180) and in March 2015 (n = 180). The identification of *A. hydrophila* by PCR showed the prevalence since 3.33% to 46.66%. The most common macroscopic lesions were hemorrhage and splenomegaly, and bacteria colonies, coagulative necrosis, hemorrhage, inflammatory process, melano-macrophages, and vacuolar degeneration were microscopic. The pathological and histopathological findings showed the presence of an infectious disease, and employing the molecular technique, it was possible to identify that the analyzed fishes had *A. hydrophila*. Thus, producers should utilize this information using histopathology and molecular techniques in tilapia to reduce economic losses and avoid disease in consumers.

Keywords *aeromonosis*, aquaculture, fish diseases, diagnostic, public health

Introduction

The aeromonads are Gram-negative, rod-shaped, facultative anaerobic, nonspore forming bacteria that are autochthonous and widely distributed in aquatic environments [1]. These bacteria, mainly *Aeromonas hydrophila*, have emerged as a foodborne pathogen of extreme importance [1-2]. *Aeromonas spp.* have been linked to both food and water-borne diseases in different parts of the world especially developing countries due to poor hygiene and poor quality water [3]. Infections in humans with bacteremia [4], respiratory tract infections [5], gastroenteristrics [6], septicemia [7], urinary tract infection [8], and traveler's diarrhea [9] have been associated with Aeromonads. *Aeromonas hydrophila* is an important cause of zoonotic diseases (i.e., diseases that can be spread from animals to humans and vice versa) [10]. In fishes, it is considered as a significant pathogen causing the motile aeromonad septicemia (MAS), also known as epizootic ulcerative

syndrome (EUS) [11]. The symptoms of *A. hydrophila* infections include swelling of tissues, dropsy, red sores, necrosis, ulceration, and hemorrhagic septicemia [12]. This bacterium has been found in several fish species, including Nile Tilapia [13-15]. *A. hydrophila* has been causing outbreaks in fish farms with high mortality rates, resulting in severe economic losses to the aquaculture industry worldwide [16-17].

Aeromonads possess a wide range of virulence factors that enable them to evade the host's defense system, spread, and eventually killing the host. Among these factors, there are different toxins and enzymes, including Lipase (Lip), Serine protease (Ser), Aerolysin (Aer), Cytotoxic enterotoxin (ACT) and temperature-sensitive protease, Epr (CAI) [13]. These bacteria have attracted the attention of researchers because of its ability to grow at cold temperatures [1]. *Aeromonas spp.* are able to survive and multiply at low temperatures in a variety of food products and can produce virulence factors even at these low temperatures [18-19]. Thus, most cases of illness in humans are associated with aquaculture products or long-term refrigerated ready-to-eat foods. Multiple resistances to some antibiotics has occurred in many strains of the pathogen, and thus, it has become a problem to cure intestinal disorders in human [1].

Due to the importance of *A. hydrophila* as a fish pathogen and as an agent of emerging foodborne diseases, representing a serious public health concern, the aim of this study was to detect this bacterium by polymerase chain reaction (PCR) in Nile tilapia (*Oreochromis niloticus*) of cage fish farms localized in hydroelectrical reservoirs of São Paulo state, Brazil. In addition, we described the lesions found in positive fish by necropsy and histopathology.

Methodology

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 gave their consent for the use of their fish for detecting pathogens and other analysis, which are not presented in this paper.

Sampling

Oreochromis niloticus specimens were randomly sampled from six fish farms from three different reservoirs in São Paulo State, Brazil, in November 2014 (n = 180) and in March 2015 (n = 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) (Table 1). Fish farm 1 is in the Paranapanema River Basin, fish farms 2-4 are in the Tiete River Basin, and fish farms 5-6 are in the Grande Paraná River Basin. 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-cm³ portion of each tissue was fixed in 10% neutral buffered formalin followed by the processing using standard histological techniques and embedded in paraffin. Hematoxylin and eosin were used for staining.

Bacterial isolation

Six *O. niloticus* with ulcers in the head and skin were used for bacterial isolation. During necropsy, kidney was swabbed for culturing in MacConkey agar and incubated at 37°C overnight. Identification was perfomed by PCR and sequencing.

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

Fish Farm	N	Weight¹ (g) ($\overline{\text{X}}$)	Weight² (g) ($\overline{\text{X}}$)	Size¹ (cm) ($\overline{\text{X}}$)	Size² (cm) ($\overline{\text{X}}$)
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**

¹ *First sampling;* ² *Second sampling.*

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. Colorless colonies were isolated and submitted to deoxyribonucleic acid (DNA) extraction. The DNA extraction for tissues was performed using the Wizard® SV Genomic DNA Purification System kit (Promega Corporation®) according to the manufacturer's instructions. For bacterial extraction, DNeasy® Blood & Tissue Kit (Qiagen®) was used according to manufacturer´s instructions. The DNA was eluted in elution buffer (nuclease-free water) and kept at -20°C. Purity and quantification of extracted DNA was measured using a 260/280 absorbance rate in a Nanodrop 2000c (Thermo Fisher Scientific®). Only the DNA samples with a ratio of >1.7 (260/280 rate) were used in this study.

The colonies extracted were submitted to PCR with universal primers designed by Weisburg et al., [21] that recognize 16S rRNA region. For this purpose, the reaction mixture consisted of 10 µL of Gotaq qPCR Mastermix 2X (Promega), 10 pmol of each primer (Univ16Sf and Univ16r) and 3 µL of DNA was prepared and finally adjusted to 20 µL by adding nuclease free water. The reaction consisted of an initial denaturation step of 95°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 54°C for 1 min, and 72°C for 1 min, ended with the finalextension at 72°C for 10 min and a hold at 22°C. The 1507 bp amplicons were purified with an Ilustra Microspin™ S-400 HR Columns Kit (GE Healthcare®) according to the manufacturer's instructions for the identification by Sanger sequencing. For this, 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). Further, sequences were identified by similarity analysis using BLAST (Basic Local Alignment Search Tool) algorithm.

Later, all samples from fish were submitted to a new PCR with specific primers for *Aeromonas hydrophila* that recognize ascV gene according to Carvalho-Castro et al., [22]. The reaction mixture consisted of 10 µL of Gotaq qPCR Mastermix 2X (Promega), 10 pmol of each primer (ascV sense and ascV antisense), 3 µL of DNA, and nuclease free water to adjust to 20 µL. The reaction consisted of an initial denaturation step of 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 58°C for 90 sec, and 72°C for 1 min, finishing with terminal extension at 72 °C for 5 min and a hold at 22 °C. The 890 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 as described above.

The nucleotide sequence of the reaction with universal primers of approximately 1100 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 *A. hydrophila* detected by PCR was calculated for each fish farm sampled in the two sampling times (November 2014 and March 2015). The occurrence of lesions was observed (necropsy and histopathology) and positive PCR results were also analyzed. All statistical analyses were performed in Statistic v. 10 (Stat Soft 2011) [23] and visualized in GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Results and Discussion

During necropsy, many lesions were observed, which were compatible to bacterial infection, such as ocular edema, splenomegaly, and ulcer of the skin (Table 2). The fishes that were positive by PCR for A. hydrophila presented the hemorrhage and splenomegaly as the most common lesions.

Table 2. Prevalence of macroscopic lesions observed in *Oreochromis Niloticus* positive for *Aeromonas hydrophila* by PCR

Lesions	A	B	C	F
Hemorrhage	100.00 (8/8)	33.33 (1/3)	66.66 (2/3)	9.09 (1/11)
Ocular edema	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)

Oreochromis niloticus with aeromonosis could present hemorrhagic patches, dark discoloration in skin, and congestion of internal organs [13]. In an experimental infection with *A. hydrophila* in *O. niloticus*, cutaneuous hemorrhage at the base of all fins and in the mouth, ascites with serobloody fluid, and exophthalmia [22] was observed. However, in this study besides hemorrhage in skin, ulcers of the skin were also visualized as the most common lesions. According to Noga [20], ulcers appears after the progress of skin lesions and can lead to exophthalmos, as observed in samples positive for this bacterium.

In histopathology analysis, processes that suggest bacterial infection were also observed. For the positive animals for *A. hydrophila*, bacteria colonies, coagulative necrosis, hemorrhage, inflammatory process, melano-macrophages , and vacuolar degeneration were the most common lesions (Table 3).

Coscelli et al., [24] performed an experiment with turbot (*Scophthalmus maximus* L.) that was challenged with *A. salmonicida*, which presented diffuse infiltrates of inflammatory cells, mainly composed by monocyte and macrophages on the connective tissue of coelomic cavity, vascular congestion, colonies of bacteria, hemorrhage and necrosis. As mentioned by these authors, we also detected these lesions (Table 3) as granulomas, degeneration, and eosinophils cells. As observed in this study, Harikrishnan et al., [25] also observed granulomatous inflammation. It was an important point because onlt this research describes this kind of lesion, which could indicate that these granulomas could be a sign that other disease causing bacteria are also present like *Mycobacterium* or *Francisella,* and suggesting the co-infection. The identification of *A. hydrophila* by PCR showed prevalence since 3.33% to 46.66%. It can be noted in Figure 1 that in the second sampling of fish farm F, an increase in the number of infected animals occurred by this species of bacteria. Jimoh and Jatau [26] detected 47%, while Balaji et al., [27] detected 41.7% of *A. hydrophila* in *Oreochromis*, that was similar to our study. However, we observed lower occurrence that could be due to the water quality and handling of the animals without causing any stress or lesions in the skin and the place where this bacterium enters in the body of the fish.

Although in this study water quality parameters were not evaluated, the difference in the prevalence from fish farms (Figure 1) could be due to the immunity of the fishes, since stress causes immunosuppression and becomes susceptible to infections.

This affirmation was supported by Janda and Abbott [28], which explains that immunosuppressed fish by spawning or environmental triggers, such as high temperatures or low water levels are more susceptible to *Aeromonas*.

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

| Lesions	A	B	C	F
Bacteria colonies	100.00 (8/8)	66.66 (2/3)	0.00 (0/3)	100.00 (11/11)
Calcification necrosis	0.00 (0/8)	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	12.50 (1/8)	0.00 (0/3)	33.33 (1/3)	18.18 (2/11)
Eosinophils cells	87.50 (7/8)	33.33 (1/3)	0.00 (0/3)	18.18 (2/11)
Granulomes	0.00 (0/8)	33.33 (1/3)	66.66 (2/3)	36.36 (4/11)
Hemorrhage	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)
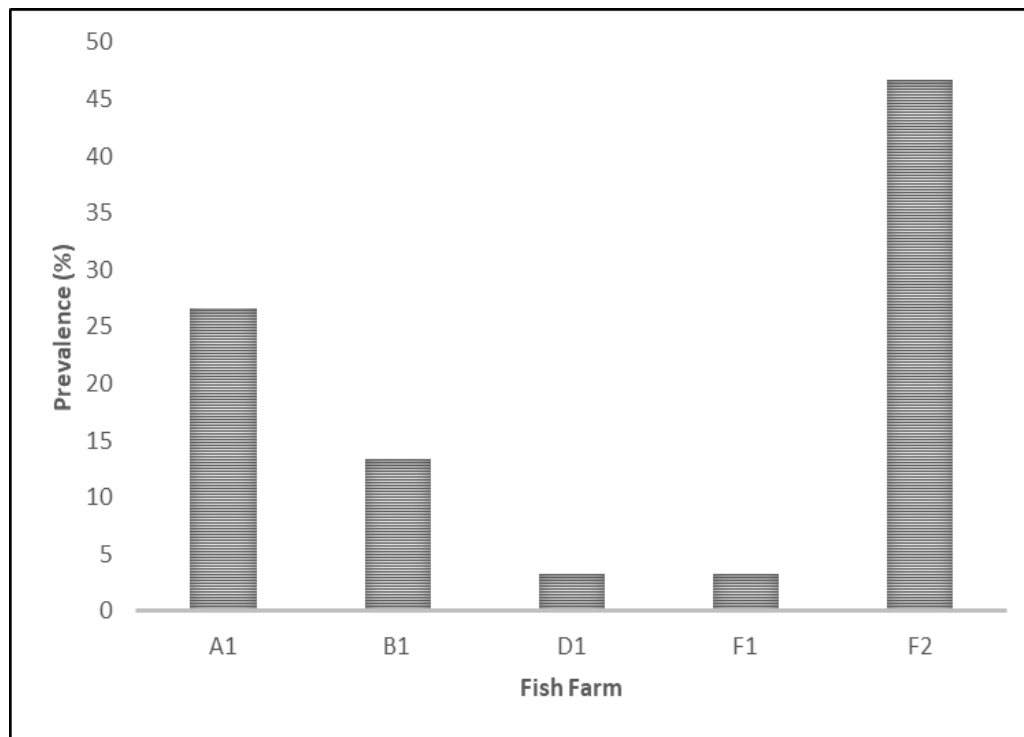
Figure 1. Prevalence (%) of *Aeromonas hydrophila* by PCR in *Oreochromis niloticus*

After sequencing and analysis, our sequence was similar (99% of identity) to *Aeromonas hydrophila* (genbank: AM992197), confirming that the bacteria isolated in this study was from this species. In the megablast search (GenBank) more than hundred sequences with identity of the 96% (the closest) with the sequence of this study were found. Figure 2 has shown the bioinformatics study results with fourteen sequences found with 96% ID and three sequences of *A. hydrophila* found in Brazil. According with the dendrogram, the closest sequence was the *A. hydrophila* TGDY isolated in China from *Betta splendens*. For *A. hydrophila* found in Brazil have 90 and 91% identityand both were found in Tilapia.
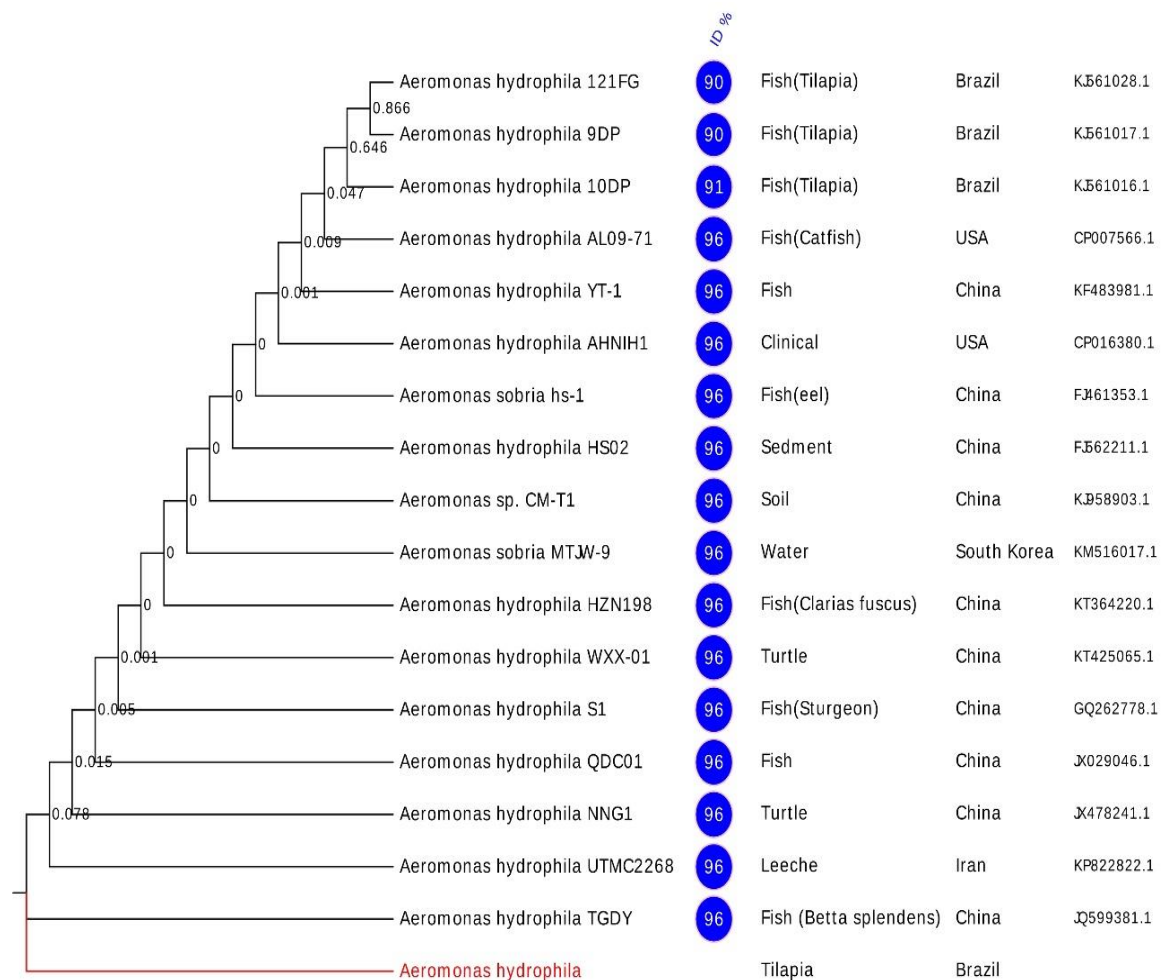

Figure 2. Dendrogram representing the bioinformatics study. The sequence in red was found in this study. The dendrogram shows respectively for each isolate: the identity with the sequence of this study, the host and country from which they were isolated and accession number from Genbank.

In the bioinformatics study, we observed that *Aeromonas spp.* similar to that found in this study were from different country, organisms and environment, but the majority were found in different fish species. Among these bacterial sequences, the most important is the *A. hydrophila* AL09-71 that was responsible for a MAS disease outbreak in 2009 in West Alabama [29], where it alone led to an estimated loss of more than 3 million pounds of food size channel catfish. Virulence studies have revealed that AL09-71, is highly virulent to channel catfish, killing the fish within 24 h post exposure [29-30]. One among these sequences was isolated from a patient, the *A. hydrophila* AHNIH1 that carried a 143-kb plasmid (pASP-135), with a bla$_{KPC-2}$ gene, TEM β-lactamase, genes encoding resistance to aminoglycosides, chloramphenicol, fluoroquinolones, macrolides, and mercury [31]. In Brazil, the closest sequence with our

study were found in Tilapia by Sebastião et al., [32], where several genera related to the pathogenic bacteria were found and among 178 bacterial isolates, *Aeromonas sp*. were with higher frequency(31%). Although in this study, we did not evaluate the presence of *Aeromonas* in fish fillets, there is an increased risk for human consumption of raw *O. niloticus*, since muscle was added to the pool in the samples analyzed for molecular tests, which had positive results (Figure 1).

Conclusion

The results found in this study by pathological and histopathological techniques showed bacterial infection in the analyzed fishes, which was also confirmed by the molecular investigation. Since *Aeromonas* is a zoonotic bacteria, it is suggested that producers should utilize the histopathological and molecular techniques to check the presence of this infectious agent to guarantee public health and avoid economic losses.

Acknowlegments

Conflict of interest

The authors declare that they have no conflict of interest.

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