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Lethal dose and clinical signs of *Aeromonas hydrophila* in *Arapaima gigas* (Arapaimidae), the giant fish from Amazon

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ABSTRACT

Aeromonas hydrophila is causing substantial economic losses in world aquaculture. This study determined the tolerance limit ($LD_{50\text{-}96h}$) of *A. hydrophila* in *Arapaima gigas*, and also investigated the clinical signs after intradermal inoculation. *Arapaima gigas* fingerlings were inoculated intraperitoneally with 0 (control), 1.0×10^5, 1.0×10^6, 1.0×10^7, 1.0×10^9 and 1.0×10^{10} CFU/mL of *A. hydrophila* for the determination of $LD_{50\text{-}96h}$, which was 1.8×10^8 CFU/mL. In another trial with intradermal inoculation of 1.8×10^8 CFU/mL *A. hydrophila*, there was a 91.6% of mortality between 8 and 23 h, and several clinical signs were found. As follows: depigmentation in the tegument, lesions in the tail and fins, loss of balance, reduction of respiratory movements, hemorrhagic foci, necrotic hemorrhages in the kidney, liver and swim bladder, splenomegaly, ascites in the abdominal cavity and hyperemia, enlargement of the gall bladder, among other clinical signs observed. The results showed that *A. gigas* has a relative tolerance to *A. hydrophila* when compared to other Neotropical fish species.

1. Introduction

Aquaculture strives to produce large quantities of fish in biological and economically efficient way, but infectious diseases are one of the major limiting factors for production (Plumb et al., 2011; Silva et al., 2012; Marinho et al., 2013) and productivity. Therefore, diseases prevention is of extreme importance to the aquaculture industry of fish as the *Arapaima gigas* Schinz, 1822 (pirarucu). This Arapaimidae species is known as the giant fish from Amazon because it can measure up to 3 m in length. This has good tolerance to the high densities of storage and its robustness in handling (Cavero et al., 2003; Araújo et al., 2009; Marinho et al., 2013). However, during the early stages of *A. gigas* culture, it encounter many problems with bacterial diseases which often cause high economic losses for fish farming due to high mortality rates.

Aeromonas hydrophila is an aquatic bacterium that can be isolated in estuarine waters, marine and freshwater, as well as in the intestinal tract of farmed fishes (Lallier and Higgins, 1988; Aoki, 1999; Janda and Abbott, 2010). This pathogen causes ulcerations, haemorrhagic focus, epizootic ulcerative syndrome, and erosion of the fins in farmed and wild fish. Its pathogenicity is mediated by different extracellular proteins such as aerolysin, lipase, chitinase, amylase, gelatinase, hemolysins, enterotoxins in addition to type III secretion system (TTSS); a specialized protein, whose role is to export virulence factors directly into the host cells, subverting the normal functions of the host for the benefit of invasive bacteria. The ADP-ribosylating toxin that leads to the interruption of the NF-kB pathway, cytoskeleton damage and apoptosis are characterized in *A. hydrophila* (Aoki, 1999; Janda and Abbott, 2010; Plumb et al., 2011; Carraschi et al., 2012; Doan, 2013; Silva et al., 2012; Song et al., 2014; Stratev and Vashin, 2014).

It has recognized that *A. hydrophila* is a pathogenic agent not only for fish, amphibians, reptiles, but also for different species of mammals including man (Lallier and Higgins, 1988; Aoki, 1999; Janda and Abbott, 2010; Plumb et al., 2011). In fishes, many

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infectious diseases display chronic characteristics that include clinical signs that persist for weeks, during which time the mortality rate increases gradually and the cumulative mortality can be high (Plumb et al., 2011).

The bacteriosis is dependent on the number of bacteria, whose concentration is variable according to the species infected and factors such as virulence, route of inoculation and temperature (Plumb et al., 2011; Carraschi et al., 2012; Song et al., 2014). Studies of experimental infection are necessary for each species, allowing the development of protocols for the prophylaxis and treatment that are more efficient against dangerous pathogen for fish farming. The objective of the current study was to determine the tolerance limit ($LD_{50\text{-}96h}$) of *A. hydrophila* to *A. gigas*, and the clinical signs after intradermal inoculation of the lethal concentration, to be applied in challenge tests.

2. Materials and methods

2.1. Fish and acclimatization

The fingerlings of *A. gigas* (38.8 ± 7.1 g and 29.6 ± 7.9 cm) were purchased from a commercial fish farm and were acclimated in the Laboratory of Aquaculture and Fisheries, Embrapa Amapa, Macapá (Brazil) in tanks of 1000 L of water. During the nursery, fish were fed with extruded feed containing 45% crude protein (CP) and, subsequently with extruded ration containing 40% CP.

2.2. hydrophila *and preparation of the culture medium*

A. hydrophila (American Type Culture Collection 7966 (ATCC 7966) was obtained at the National Reference Laboratory for Bacterial Enteroinfections (LABENT) of the Oswaldo Cruz Institute (FIOCRUZ), Rio de Janeiro (Brazil), belonging to the Collection of Bacterial Enteropathogens and maintained in cryopreservation.

The liquid culture medium Brain Heart Infusion (BHI – Fluka Analytical – Sigma AldrichTM, St. Louis, MO, USA) was prepared according to the instructions of the manufacturer by dissolving 37 g in 1 L of distilled water. The sterilization was processed by autoclaving at 121 °C for 15 min (Phoenix® AV 75, SP, Brazil). The GSP cultivation agar medium (GSP Agar – Fluka Analytical – Sigma AldrichTM, St. Louis, MO, USA) was prepared according to the manufacturer's instructions by dissolving 45 g in 1 L of distilled water and sterilized by autoclave at 121 °C for 15 min. After removal and cooling between 45 and 50 °C, 100,000 International Units of Penicillin G per liter (UI/L) were added followed by homogenization and distribution in 90 mm sterile disposable Petri dishes.

2.3. Preparation of hydrophila *suspensions*

Bacterial suspensions were prepared by transferring a heave containing three to five colonies of *A. hydrophila* isolated in Petri dishes containing the GSP agar medium after 24 h of cultivation at 35 °C to Falcon tubes containing 10 mL of BHI broth and reincubated for 18 h in a bacteriological incubator (Olidef® CZ-ECB1 Linea, SP, Brazil).

After incubation of the bacterial suspensions with the culture, logarithmic growth phase was measured by turbidity caused by bacterial growth; densitometer Densichek™ (BioMerieux, Marcy l'Etoile, France) was designed to measure the optical density of a suspension of microorganisms. The values were processed in nephelometric units of McFarland and adjusted to the respective concentrations of the number of tubes from 0.5 to 7 of that scale. To obtain concentrations exceeding the reading capacity of Densichek™, bacterial inocula were adjusted and subsequently, suspensions from 10 mL were centrifuged at 1000*g* for five minutes for the formation of pellet with ten times concentration. Once standardized bacterial concentrations and, respected the interval of up to 15 min after adjustment, fish were inoculated with the bacterial suspension.

2.4. Lethal dose ($LD_{50\text{-}96h}$) of hydrophila *to* A. gigas

Ninety specimens of *A. gigas* (353 ± 110 g and 39 ± 27 cm) were distributed in six tanks of 100 L (n = 15 fish/tank), and maintained in fasting during the 96 h previously to the LD_{50} trial. Fish were inoculated intraperitoneally with 1.0 mL of *A. hydrophila* suspension with the following concentrations of the bacterium: 0CFU/mL (control using 1.0 mL of NaCl 0.85%), 3.0×10^5 CFU/mL, 3.0×10^6 CFU/mL, 3.0×10^7 CFU/mL, 1.0×10^9 CFU/mL and 1.0×10^{10} CFU/mL. The $LD_{50\text{-}96h}$ was estimated using the Spearman-Karber method (Hamilton et al., 1977).

This study was conducted in accordance with the principles adopted by the Brazilian College of Animal Experimentation (COBEA) and was approved by the Ethics Committee on Animal use (CEUA/UFAC: 08/2014).

2.5. Intradermal inoculation of hydrophila *in* A. gigas

Fingerlings of *A. gigas* (320 ± 120 g and 38 ± 28 cm) were acclimated for three days in aquaria of 100 L of water and kept under constant aeration, for intradermal inoculation of *A. hydrophila* in the region of the caudal peduncle exactly on the lateral line. The bacterial suspension was adjusted to 1.0×10^8 CFU/mL and, once standardized as previously described, and observing the range for up to 15 min after adjustment, fishes were inoculated. Fishes were divided into two treatments, 0 CFU/mL (inoculated control with 0.5 mL of NaCl solution 0.85%) and 0.5 mL of the bacterium suspension of 1.0×10^8 CFU/mL and three repetitions each, were inoculated with the bacterial suspension intradermal, to investigate for clinical signs of bacteriosis. The inoculation of fish in the control group preceded those of the group that received the inoculation of *A. hydrophila* to avoid possible cross-contamination. Observations were made of behavioral changes, morbidity and mortality of the fish after inoculation for a period of 24 h.

2.6. The water quality in the assays with hydrophila

During the assays, the static water system and constant sandblasting was used. On a daily basis, a portion of the volume of water tanks and aquariums was changed. The levels of dissolved oxygen (6.0 ± 0.1), temperature (27.6 ± 0.7 °C), pH (7.2 ± 0.8), were measured daily, using multi-parametric probe (Hanna, mod. HI 96715@) and the levels of total ammonia (4.9 ± 2.9 mg/L) and nitrite (0.03 ± 0.03 mg/L) were measured using digital apparatus (Hanna, mod. HI 96715@).

3. Results

Mortality was not observed after 96 h of inoculation with 1.0×10^5 and 1.0×10^5 CFU/mL of *A. hydrophila*. However, first

Table 1
Mortality of *Arapaima gigas* during the $DL_{50\text{-}96h}$ of *Aeromonas hydrophila*.

Pathogen concentration (UFC/mL)	24 h	48 h	72 h	96 h	%
0	0	0	0	0	0
1.0×10^5	0	0	0	0	0
1.0×10^6	0	1	0	0	6.6
1.0×10^7	0	3	0	0	20.0
1.0×10^9	12	0	0	0	80.0
1.0×10^{10}	13	0	0	0	86.0