LD$_{50}$ of the bacteria Aeromonas hydrophila to matrinxã, *Brycon amazonicus*

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ABSTRACT

In order to determine the lethal dose (96-h LD$_{50}$) of the bacteria *Aeromonas hydrophila* to matrinxã, *Brycon amazonicus*, to be applied in challenge tests, 90 fish (63.23 ± 6.39 g) were divided into five treatments, with different bacterial solutions: T$_1$ - Control (0.9% NaCl saline solution); T$_2$ (4 x 10^{11} cells/ mL); T$_3$ (5 x 10^{11} cells/ mL); T$_4$ (1.36 x 10^{12} cells/ mL) and T$_5$ (3.06 x 10^{12} cells/ mL). Fish were previously anesthetized with benzocaine (60 mg L^{-1}), inoculated in the peritoneal cavity with the bacterial suspensions and then distributed into fifteen 80-L test chambers, where the water variables were monitored and fish mortality was observed. The experiment was randomly designed in three replicates and the 96-h LD$_{50}$ was estimated according to the trimmed Spearman-Karber method. Water quality variables remained within adequate ranges for fish health and performance. Fish mortality rate increased with the bacterial concentrations of *A. hydrophila* (T$_1$ = 0%; T$_2$ = 16.66%; T$_3$ = 44.44%; T$_4$ = 72.22% and T$_5$ = 100%), and the first mortalities were observed after 57 h, although the signs of the bacterial infection were already observed 24 h after the inoculation. The results indicate that the 96-h LD$_{50}$ value of *A. hydrophila* to matrinxã is 6.66 x 10^{11} cells/ mL.

KEYWORDS: *Aeromonas hydrophila*, 96-h LD$_{50}$, matrinxã, tropical fishes

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RESUMO

Para determinar a dose letal (DL$_{50}$ 96-h) da bactéria *Aeromonas hydrophila* para o matrinxã, *Brycon amazonicus*, com aplicabilidade para testes de desafio, foram utilizados 90 peixes (63,23 ± 6,39 g), divididos em cinco tratamentos, com diferentes soluções bacterianas: T$_1$ - Controle (solução salina 0,9% NaCl); T$_2$ (4 x 10^{11} células/ mL); T$_3$ (5 x 10^{11} células/ mL^{-1}); T$_4$ (1,36 x 10^{12} células/mL^{-1}) e T$_5$ (3,06 x 10^{12} células/ mL^{-1}). Os peixes foram previamente anestesiados com benzocaína (60 mg L^{-1}), inoculados na cavidade peritoneal com as suspensões bacterianas e distribuídos em 15 aquários de vidro de 80 L de capacidade, com aeração constante. O experimento teve duração de 96 h, no qual foram monitoradas a mortalidade e a qualidade da água. O delineamento experimental foi inteiramente casualisado com três réplicas e a DL$_{50}$ 96-h foi estimada de acordo com o método Spearman-Karber. Durante o experimento os parâmetros físico-químicos da água permaneceram dentro das condições consideradas adequadas para o desenvolvimento e saúde dos organismos aquáticos. A mortalidade dos peixes aumentou nas concentrações crescentes de *A. hydrophila* (T$_1$ = 0%; T$_2$ = 16,66%; T$_3$ = 44,44%; T$_4$ = 72,22% e T$_5$ = 100%), contudo, as primeiras mortalidades ocorreram em 57 h após a inoculação das concentrações bacterianas, sendo observados os primeiros sinais de infecção em 24 h após a inoculação. Os resultados indicam que o valor da DL$_{50}$ 96-h da bactéria *A. hydrophila* para o matrinxã foi igual a 6,66 x 10^{11} células/mL de solução salina.

PALAVRAS-CHAVE: *Aeromonas hydrophila,* DL$_{50}$ 96-h, matrinxã, peixes tropicais

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INTRODUCTION

Matrinxã, a native Amazonian fish, stands out as one of the greatest economic valuable species with enormous potential for intensive breeding (Mendonça 1994; Graef 1995; Val and Honczaryk 1995; Guerra *et al.* 1996; Honczaryk 2000; Pezzato *et al.* 2000; Fim 2002; Brandão *et al.* 2005). Although well adapted to captivity, this species is very sensitive to handling and transport stress, being very agitated and disturbed, especially in occasions where the space is reduced (Inoue *et al.* 2003). Due to this extreme activity, scale and mucus losses are observed, facilitating the occurrence of diseases which can cause major losses to the fish culture (Inoue *et al.* 2003).

Although some researchers have already described techniques to minimize the stress effects on matrinxã (Carneiro and Urbinati 2001; Roubach *et al.* 2001; Urbinati and Carneiro 2001; Inoue *et al.* 2002; Inoue *et al.* 2004; Urbinati *et al.* 2004; Inoue *et al.* 2005), their application can only minimize the stress responses during the stressor agent and, consequently, the mortality rates. Therefore, the adverse effects of the stress responses after stressor agents are still an issue and must be overcome.

Recently, the demand for products which can prevent fish diseases and improve immunological resistance in intensive culture conditions has been an alternative to the improvement of handling techniques (Chen and Ainsworth 1992; Siwicki *et al.* 1994; Sakai *et al.* 1996; Findlay and Munday 2000; Sakai *et al.* 2001; Sahoo and Mukherjee 2003; Rao and Chakrabarti 2005; Chakrabarti and Rao 2006). In order to evaluate the efficiency and security of new aquaculture products, challenge tests have been developed, mainly, using pathogenically bacteria as a stressor agent, due to the easy techniques and high quality results. These challenge tests are described in the literature using intraperitoneally injected bacteria, in order to evaluate the efficiency of tested products in controlling the organism responses to an infectious agent or even in preventing fish mortality (Kim *et al.* 2001; Jain and Wu 2003; Kodama *et al.* 2007; Rairakhwada *et al.* 2007; Sahu *et al.* 2007).

For this propose, bacteria such as *Aeromonas hydrophila* are commonly used as they are worldwide distributed and are responsible for considerable losses in aquaculture. Its natural habitat is the decomposing organic matter in the water, being as well present in healthy fish intestinal flora. *A. hydrophila* can cause hemorrhagic septicemia in stressed fish (Moraes and Martins 2004), which occurs mainly in environments with high temperature water and elevated organic matter in association with other stressor factors, such as high stocking densities, traumas due to inappropriate handling, hypoxia, nutritional deficiencies and infections (Costa 2004).

Using these bacteria as an efficiency indicator for new products that can minimize stress in intensive fish culture systems can contribute to the improvement of the aquaculture in the Amazon region. Therefore, the objective of the present work is to determine the tolerance limit (96-h LD$_{50}$) of the bacteria *Aeromonas hydrophila* to matrinxã, *Brycon amazonicus,* to be applied in challenge tests.

MATERIAL AND METHODS

Matrinxã fingerlings were obtained from local producers and transferred to the Aquaculture Department from National Institute of Amazonian Research (INPA), in Manaus, Brazil, where they were kept in 150 m²-escavated ponds. Fish were fed 40% crude protein extruded feed until they reached mean weight of 60 g.

Bacteria origin and the bacterial solutions prepare

A. hydrophila bacterial cepa used in this experiment was obtained from the UNESP Aquaculture Center – CAUNESP – Jaboticabal/SP, and maintained at the Microbiology Laboratory/CPTA/INPA. To prepare the experimental solutions the following procedures were performed: previously, the sample was reactivated by immersing in nutritive solution and incubation for 24 hours at 30 °C. Next, the material was plated on Müeller-Hinton Agar by the spread-plate technique and incubated for the same period and temperature. Later, a concentrate solution was made using a sterile 0.9% NaCl saline solution, of which 10 µL was collected to determine bacterial concentration by using a Neubauer chamber and an optical microscope. Finally, four diluted solutions were made: 4×10^{11} cells/ mL, 5×10^{11} cells/ mL, 1.36×10^{12} cells/ mL and 3.06×10^{12} cells/ mL, which were intraperitoneally inoculated in fish.

Experimental design

Ninety fish (63.23 ± 6.39 g) were divided into five treatments, in triplicates, being each treatment composed of 18 fish: T_1 = Control (0.9% saline solution); T_2 = 4×10^{11} cells/ mL; T_3 = 5×10^{11} cells/ mL; T_4 = 1.36×10^{12} cells/ mL and T_5 = 3.06×10^{12} cells/ mL.

Fish were kept in fasting for 24 h before the beginning of the experiment. For the inoculation, fish were previously anesthetized with benzocaine (60 mg L^{-1}), inoculated in the peritoneal cavity with 0.1 mL the different bacterial suspensions and then distributed in fifteen 80-L test chambers equipped with air compressors, in a semi-static system. The experiment lasted for 96 hours, in which fish mortality was observed and water variables were monitored.

Water quality monitoring

The water quality of each test chamber was monitored daily (9:00 h) to avoid any alteration that would hinder the

bioassay results. After the water sampling, a third of each aquarium volume was changed to avoid water deterioration.

Dissolved oxygen (DO), temperature and electric conductivity were determined using a digital DO-meter (YSI - Yellow Springs Instruments - model 85/10); pH was measured using a pH-meter (YSY, model 60/10). Water samples were collected and the colorimetric method was applied to determine total ammonia ($NH_3 + NH_4^+$) and nitrite (NO_2^-) concentrations, according to Verdouw *et al.* (1978) and Boyd and Tucker (1992), respectively. Non-ionized ammonia values were calculated according to Kubitza (2003). Total alkalinity, total hardness and oxygen dioxide (CO_2) were determined according to Boyd and Tucker (1992), however, CO_2 was determined using an adaptation for minimum oxygen contact.

Statistical Analysis

Water quality data are reported as mean ± SD. The mean values of different treatments were compared by using the ANOVA analysis. The differences were considered to be significant at $p<0.05$ using the Tukey test. The 96-h LD$_{50}$ (lethal dose to 50% of a population) was estimated according to the trimmed Spearman-Karber method (Hamilton *et al.* 1977).

RESULTS

Water quality

Water quality parameters in the tanks during the experiment are shown in Table 1. Those parameters (DO, temperature, conductivity, pH, alkalinity, hardness and CO_2) did not show any statistical difference throughout the experimental period and were within the limits considered to be suitable for health maintenance and production performance of tropical fish (Kubitza 2003). However, ammonia and nitrite concentrations presented daily alterations according to the different treatments,

being the higher values observed in treatments of which fishes were infected with the higher bacterial concentrations.

Fish mortality and lethal dose (LD$_{50}$) of *A. hydrophila* to matrinxã

No mortality was observed during the 96-h experiment for treatment T1 (Control). However, a crescent mortality increase was observed with the increase of the bacterial concentration (Table 2).

Table 2 - Matrinxã, *B. amazonicus*, mortality after 96 h of intraperitoneal inoculation of different *A. hydrophila* concentrations. T$_1$ = Control (saline solution 0.9%); T$_2$ = 4 x 10^{11} cells/mL; T$_3$ = 5 x 10^{11} cells/mL; T$_4$ = 1.36 x 10^{12} cells/mL e T$_5$ = 3.06 x 10^{12} cells/mL.

Treatment	Number of fishes (0h)	Number of fishes (96h)	Mortality (%)
T$_1$	18	18	0
T$_2$	18	15	16,66
T$_3$	18	10	44,44
T$_4$	18	5	72,22
T$_5$	18	0	100

Although the first mortalities were observed after 57 h of the bacterial inoculation, the first signs of bacterial infection, such as fin erosion and scales losses, were observed 24 h after the inoculation. Ulcerative lesions were also observed on the eyes and later on the abdomen and urogenital pore. Clinical signs which also preceded the death of infected animals were equilibrium loss and slower respiratory movements, as well as oftalmia and mucus excess in head region.

Using fish mortality data during the 96-h experiment, the LD$_{50}$ was determined by the trimmed Spearman-Karber method. According to the results, the lethal dose of *A. hydrophila* to 50% of a population of matrinxã is 6.66 x 10^{11} cells/ mL of saline solution.

Table 1 - Water physical and chemical parameters during a 96 hours experiment to determine the LD$_{50}$ of *A. hydrophila* to *B. amazonicus*. Values are shown as Mean ± SD. T$_1$ = Control (saline solution 0.9%); T$_2$ = 4 x 10^{11} cells/mL; T$_3$ = 5 x 10^{11} cells/mL; T$_4$ = 1.36 x 10^{12} cells/mL and T$_5$ = 3.06 x 10^{12} cells/mL.

Parameters	Treatments				
	T$_1$	T$_2$	T$_3$	T$_4$	T$_5$
Oxygen (mg/L)	5.73±0.20[a]	5.86±0.11[a]	5.83±0.13[a]	5.69±0.22[a]	5.75±0.17[a]
Temperature (ºC)	26.17±.24[a]	26.23±0.20[a]	26.23±0.23[a]	26.25±0.19[a]	26.22±0.22[a]
Conductivity (μS/cm^3)	27.92±2.93[a]	28.86±4.52[a]	29.31±6.19[a]	29.45±6.88[a]	28.64±4.18[a]
pH	7.13±0.14[a]	7.10±0.18[a]	7.05±0.22[a]	7.11±0.14[a]	7.08±0.16[a]
Alkalinity (mg CaCO$_3$/L)	13.47±0.9[a]	14.18±1.7[a]	14.73±1.8[a]	15.16±2.2[a]	13.56±1.5[a]
Hardness (mg CaCO$_3$/L)	20.02±3.1[a]	19.46±1.5[a]	20.58±2.6[a]	21.13±3.7[a]	19.32±1.9[a]
Dioxide carbon (mg/L)	11.17±2.79[a]	11.04±3.61[a]	10.83±3.54[a]	10.33±3.19[a]	11.10±2.9[a]
Total ammonia (mg/L)	1.37±0.44[b]	1.84±0.53[ab]	2.09±0.65[ab]	2.46±0.72[a]	2.50±0.68[a]
Toxic ammonia (mg/L)	0.008±0.000[b]	0.011±0.000[b]	0.012±0.000[a]	0.014±0.000[a]	0.016±0.000[a]
Nitrite (mg/L)	0.005±0.000[b]	0.005±0.000[b]	0.006±0.000[b]	0.011±0.000[a]	0.011±0.000[a]

Lines that have the same letters indicate statistical consistencies (p>0.05).

DISCUSSION

It is common to observe and record water quality parameters in fish tanks during laboratory essays, as the variation of any parameter can interfere the results (Andrade *et al.* 2006; Affonso *et al.* 2007). This control should be even more stringent when determining the tolerance of a given species to a stressor agent, in order to assure reliable results, which dictate the animal tolerance limits (Lima 2003; Avilez *et al.* 2004; Cavero *et al.* 2004). For example, studies involving *Piaractus mesopotamicus* indicate that water temperature seems to have an influence on the mortality rates caused by *A. hydrophila* and septicemia outbreaks are associated with high water temperatures (Garcia *et al.* 2009). In the present study most values recorded were within the limits considered to be adequate for the development and health of aquatic organisms, according to Kubitza (2003).

Aeromonas bacteria can attack fish fins, tegument and intestines. According to Pavanelli *et al.* (2002), these bacteria are capable of rupturing little blood vessels, resulting in ulcerative lesions in tegument with a hemorrhagic aspect, causing a reddish color on the body. In this study, besides the ulcerative lesions observed on the body, exoftalmy and mucus excess were also observed. Pavanelli *et al.* (2002) have described these manifestations as clinical signs of *Aeromonas hydrophila* infection and, according to Costa (2004), the high proliferation of these bacteria on fish intestine can cause excessive mucus liberation.

Besides the clinical signs described in this study some authors (Angka *et at.* (1995); Schlotfeldt and Alderman 1995) have observed that the infection of *A. hydrophila* can also cause necrotic hemorrhaging in internal organs, mainly kidneys and liver and fluid deposition in the abdominal cavity. Barja and Esteves (1988) have observed fin hemorrhages around the anus and intern organs, like the liver, spleen and kidney. Boijink and Brandão (2001) did not observe apparent fin and organs hemorrhages in *Rhamdia quelen*, although they have verified lesions around anus and urogenital pore. Besides the hemorrhagic lesions, Garcia *et al.* (2009) have also observed distended abdominal cavity, with clear ascites content. Secombes (1999) reported that neutrophil extracellular enzymes can cause damage to the tissues of the host, possibly contributing toward the hemorrhagic liquefaction of tissues commonly seen in bacterial infections.

The effects of *A. hydrophila* on fish can vary according to their resistance to the infection (Schlotfeldt and Alderman 1995). Santos *et al.* (1991) have determined, during seven days, the LD$_{50}$ of *A. hydrophila* to several fish species: *Salmo trutta* (2x10^5 cells/ mL), *Anguilla japonica* (>10^8 cells/ mL), *Plecoglossus altivelis* (8,6x10^4 cells/ mL), *Lepomis macrochirus* (>10^8 cells/ mL). According to these authors, the bacteria toxicity can vary with the bacterial cepa, of which the LD$_{50}$

values to *Onchorhyncus mykiss* varied between 3.2x10^4 to 3.2x10^8 cells/ mL. Boijink and Brandão (2001) have found lethal doses of *A. hydrophila* to *R. quelen* of 1.3x10^9 and 3.5x10^8 UFC mL^{-1}, when intramuscularly inoculated .

Andrade *et al.* (2006) have determined the LD$_{50}$ of *A. hydrophila* to 55 g matrinxã of 4,6x10^{11} cells/ mL. These inferior values, when compared to the results found in these experiments, can suggest that the lethal dose can also vary according to the animal size.

Analyzing the results found in this study, we suggest that matrinxã presents high tolerance to *A. hydrophila*, being the lethal concentration for 50% of the population, in 96 hours, of 6.66 x 10^{11} cells/ mL of saline solution.

ACKNOWLEDGEMENTS

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