Sodium fluorescein for early detection of skin ulcers in *Aeromonas hydrophila* infected *Piaractus mesopotamicus*

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Abstract
The increase in aquaculture global fish production has been associated with an increase in infectious diseases affecting aquacultured species.. The aim of this study was to identify ulcers in pacu (*Piaractus mesopotamicus*) with sodium fluorescein before it was possible to observe them visually. Twenty one fish with no apparent skin lesions were divided into three groups. Group 1 were injected with saline 0.65 % (Group 1), Group 2 with 6 x 10^8 CFU of *Aeromonas hydrophila* and group 3 with 6 x 10^8 CFU of *Streptococcus agalactiae* into the peritoneal cavity. All animals showed a positive reaction to fluorescein. *A. hydrophila* infected fish presented with large round marks with irregular borders, the characteristic ulcers caused by this agent. However, the other groups only displayed markings associated with handling caused by the netting procedures. We concluded that sodium fluorescein may be useful for an early and fast evaluation of cutaneous infection of *A. hydrophila* in pacu.

Introduction

The strong and continuous growth of Brazilian aquaculture in the last years (FAO, 2012) has led to an increase of fish farms, especially intensive fish farming, reducing individual production costs (Brasil, 2010). However, the stress produced by rearing fish at very high density has probably may lead to reduced resistance to infectious agents, that are widely prevalent in the water bodies the fish are reared in (Souza-Filho and Cerqueira, 2003).

Aeromonas hydrophila is one of most important pathogens in tropical fish culture (Basha et al, 2013). This bacterium produces large economic losses, partially due to the difficulty of a rapid assessment and subsequent delay of treatment. One of the first clinical signs of this disease is the appearance of skin ulcers (Karunasagar et al, 1995), which in the pre-clinical phase of ulcer formation are not visible to the naked-eye. Pacu (*Piaractus mesopotamicus*) is an emergent species in the world of aquaculture. It is a native teleost fish of the Parana–Paraguay Basin, and is of importance in South America for human consumption, angling and aquaculture (Belo et al., 2005).

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Sodium fluorescein is a dye widely used in ophthalmological procedures in mammals, mainly intravenously (Mintern et al, 1999). This is based on the chemical properties of the substance that can penetrate damaged epithelium without binding to intact tissues (Stades et al, 1999). In this work, we tested it as a marker of an early stage of ulcers in experimentally *A. hydrophila* infected *P. mesopotamicus.*

Materials and methods

21 mixed sex pacus (*P. mesopotamicus*), of average size 260 ± 25 g, without apparent cutaneous lesions, were divided into three groups (N=7). Each group were acclimatized in a 250 L-tank, aeration supply and fed commercial feed (28% crude protein), 2 times per day at 3% fish body weight and for 1 week prior to challenge.

Group 1 - control group fish were injected into the intraperitoneal cavity (ip) with saline solution 0.65%; Group 2 – were ip injected with a solution containing *Aeromonas hydrophila*, (6 x 10^8 CFU) and Group 3 – were ip injected inoculated with a solution containing *Streptococcus agalactiae* (6 x 10^8 CFU), ip.

The bacterial strains were isolated from naturally infected fish and identified based on morphological, biochemical and, molecular features (Holt et al, 1994; Vandamme et al, 1997; Borrel et al, 1997). Inoculums of 1.0mL containing approximately 1 x 10^8 colony-forming units per mL (CFU/mL) were prepared, based on previously determined LC50 dose levels for both pathogens (Iqbal et al., 1999; Salvador et al, 2013).

Seven days after inoculation, fish were anesthetized with a alcoholic solution of benzocaine (Sigma-Aldrich Laboratory, Steinheim, Germany), at a dose of 1:2000 dissolved in ethanol 98% (0.1 g/mL) (Wedemeyer, 1970, Claudiano et al., 2013) and, after that, fish were placed in an aqueous solution with 0.10 mg/mL of fluorescein sodium solution for 15 min. Thereafter, fish were placed in clean water for two min. Finally, all fish were examined under UV light in a dark room (Spectoline Bi-O-Vision Model DVM-1000R/F, United States) for the detection and characterization of lesions. The fish were killed by an overdose of anesthetic and, after necropsy, skin tissue fragments were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin (Bancroft and Stevens, 1996) and examined microscopically.

Fish that were not submitted to necropsy were returned to their tanks. After ten days, the bacteria were re-isolated from samples of aseptically collected liver and kidney. Solid medium brain-heart infusion (BHI, Difco) with 5% of sheep blood was used to isolate *S. agalactiae* (Salvador et al, 2005) and solid tryptic soy agar (TSA, Difco) (Nielsen et al, 2001) to isolate *A. hydrophila.*

Results and discussion

Experimentally inoculated animals with *A. hydrophila* showed clear differences in marking patterns compared to the control group (Figure 1). *A. hydrophila* infected fish (group 2) presented with large round marks with irregular borders, similar in size and appearance to the ulcers caused by this pathogen. Group 1 (control) showed multiple linear marking distributed throughout the skin, probably caused by netting injuries when the fish were removed from the water.

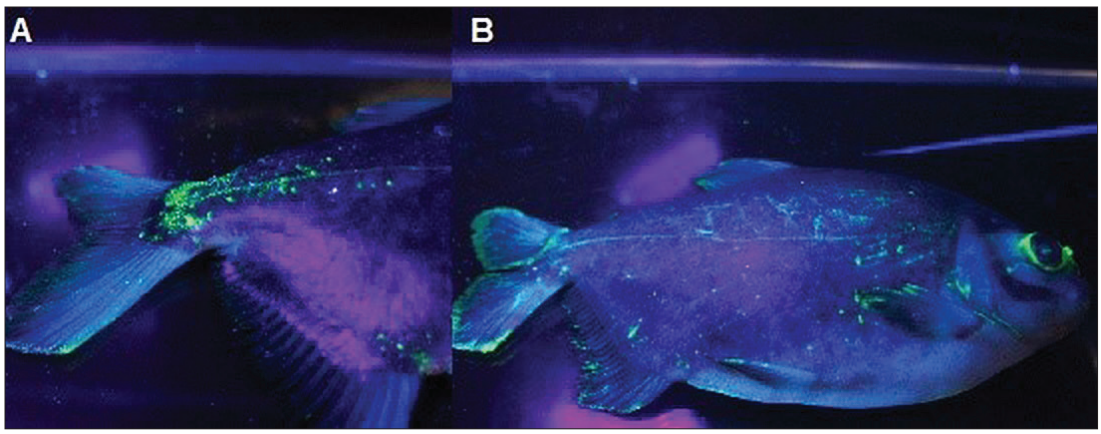

Figure 1. (A) Large round marks with irregular borders in the caudal peduncle stained with fluorescein in an *A. hydrophila* infected *P. mesopotamicus*. (B) Multiple linear marking distributed across fish skin in the control group.

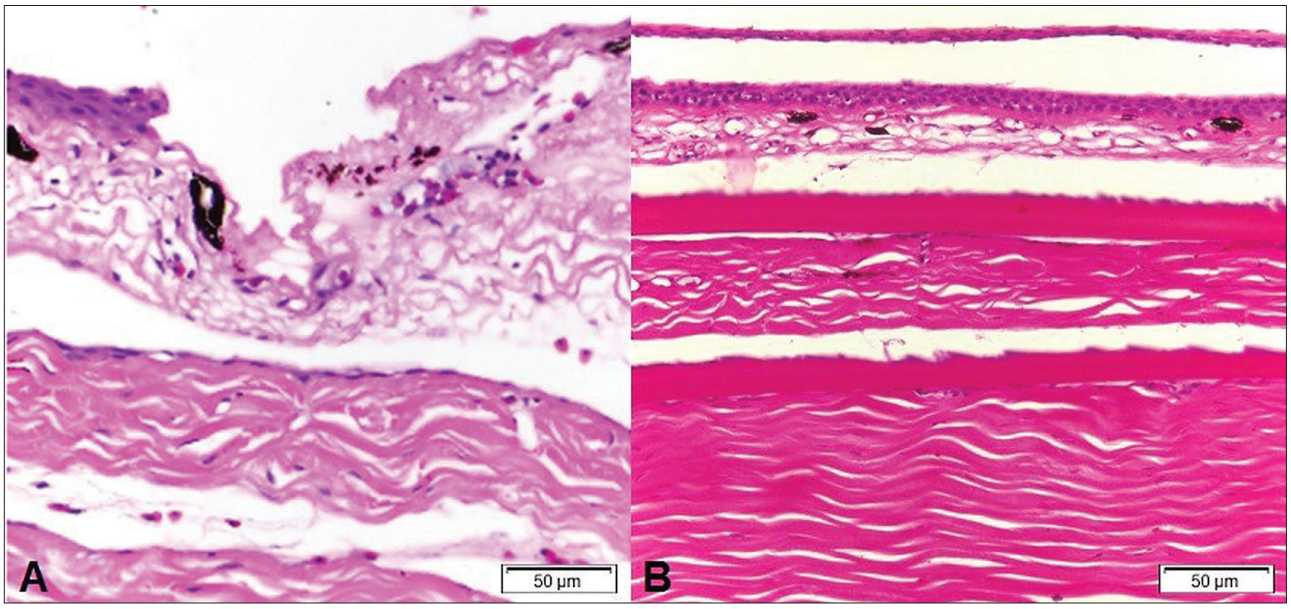

Figure 2. (A) Necrotic ulcer with loss of epidermis and dermis integrity and presence of scarce lymphocytic cells infiltrate and dissociation of collagen fibers. Bar 50 µm. HE. (Group 2). (B) Epidermis and dermis of pacu without alterations, absence of ulcers (Control Group). Bar 50 µm. HE.

Fish from group 3 also presented with multiple linear marks, similar to those observed in the control group. This was likely due to the absence of ulcers or other skin lesion in the pathogenesis of this bacterium in tropical fish (Salvador et al., 2005). However, these fish displayed a range of clinical symptoms, including erratic swimming, lethargy, exophthalmoses, corneal opacity, and unilateral or bilateral intraocular hemorrhage. These clinical signs are typically associated with streptococcal infection (Salvador et al., 2012)

The positive staining marks found in group 2 were mainly at the caudal peduncle. This position is cited by Llobrera and Gacutan (1987) and Boijink and Brandão (2004) as the preferred location for the appearance of ulcers in fish infected by *A. hydrophila*. Despite staining results, it was

not possible observe ulcers or other clinical signs with the unaided eye. Both *A. hydrophila* and *S. agalactiae* were re-isolated from kidney and liver in all infected fish in groups 2 and 3.

This technique provided an early detection of *A. hydrophila* infection in pacus, which could improve the chances of success in the treatment of this disease, and consequently lower economic losses to fish farmers. Furthermore, it is possible to measure the degree of the trauma caused by handling (Livengood et al., 2013), which would help in the improvement of fish management practices.

Histopathology revealed major differences between groups. *A. hydrophila* infected fish showed multiple foci of necrotic ulcers with loss of epidermis and dermis integrity. They were also found subepithelial and epithelial edema with separation between collagen fibers and presence of lymphocytic inflammatory cells infiltrate in the dermis (Figure 2). Ibrahem and Mesalhy (2010) found similar results in the histology of *Oreochromis niloticus* marked with sodium fluorescein.

Conclusion

Marking with sodium fluorescein allowed early observation of ulcers caused by *A. hydrophila* infection, before they could be observed with the naked eye and allowed a clear differentiation from handling-related injuries. However, this technique was not suitable for *S. agalactiae* infection.

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