










Clinical Reports

Detection of *Pseudomonas* sp. in pirarucu (*Arapaima gigas*): a case report in the Western Amazon

Detecção de *Pseudomonas* sp. em pirarucu (*Arapaima gigas*): um relato de caso na Amazônia Ocidental

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ABSTRACT

Pirarucu (*Arapaima gigas*) is one of the most important fish in Amazonian fish farming. However, information about its nutritional, zootechnical and microbiological aspects is still scarce. This is especially true for the juvenile phase due to high mortality rates caused by malnutrition, difficulties in food training, endo and ectoparasite infestations, which in turn lead to immunosuppression, favoring secondary bacterial infections that may be present due to various environmental factors (e.g., sudden temperature variations, water acidity and pollution of the aquatic environment) and the status of fish. The *Pseudomonas* sp. species studied in this work is part of the aquatic ecosystem and is considered a contaminant or invader because it infects a wide variety of aquatic species, including pirarucu. Given this assumption, the objective of the study was to report a case of *Pseudomonas* sp. in the viscera and dorsum of a juvenile pirarucu *Arapaima gigas* (SCHINZ, 1822) detected through bacteriological analysis. In the fish production chain in the Western Amazon, pirarucu is one of the most prominent fish species because of its high zootechnical performance in terms of weight gain and commercial value. However, one of the biggest obstacles in its production chain occurs during the juvenile phase, with high mortality rates caused by mainly bacterial infections, leading to economic losses in fish farming. In this study, *Pseudomonas* sp. was detected in a sample of dorsum and viscera of a pirarucu fish from a fish farm in the municipality of Ouro Preto do Oeste, Rondônia, Brazil.

RESUMO

O pirarucu (*Arapaima gigas*) é um dos peixes de maior interesse para piscicultura amazônica. No entanto, os conhecimentos sob os aspectos nutricional, zootécnico e microbiológico desse peixe ainda são escassos. Sobretudo, na fase juvenil, porque nessa fase ocorrem altas taxas de mortalidade porque são mais susceptíveis à desnutrição, dificuldades no treinamento alimentar, infestações por endo e ectoparasitas, que por sua vez, ocasionam imunossupressão, favorecendo infecções bacterianas secundárias que podem estar presentes em virtude de diversos fatores ambientais e estado imunológico dos peixes. São exemplos, variações bruscas de temperatura, acidez da água e poluição do ambiente aquático. A espécie *Pseudomonas* sp. estudada nesse trabalho faz parte do ecossistema aquático, sendo considerada como contaminante ou um invasor, porque infecta grande variedade de espécies aquáticas, inclusive o pirarucu. Diante do pressuposto, o objetivo do trabalho foi relatar o caso de detecção de *Pseudomonas* sp. em vísceras e dorso do pirarucu *Arapaima gigas* (SCHINZ, 1822) juvenil por meio de análise bacteriológica. Na cadeia produtiva do pescado na Amazônia Ocidental, o pirarucu é um dos peixes de destaque por ser uma espécie com alto desempenho zootécnico em ganho de peso e valor comercial. Porém, um dos maiores empecilhos da cadeia produtiva se encontra na fase juvenil da espécie havendo altos índices de mortalidade ocasionados por infecções principalmente de origem bacteriana levando a perdas econômicas a piscicultura. Neste estudo detectou-se *Pseudomonas* sp. em amostra de dorso e vísceras de pirarucu em uma piscicultura no município de Ouro Preto do Oeste, Rondônia, Brasil.

Palavras-chave:

Piscicultura

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Bactéria oportunista

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INTRODUCTION

Fish farming is a common practice throughout Brazil, mostly consisting of semi-intensive farming systems, including native species such as pirarucu *Arapaima gigas* (SCHINZ, 1822) in the Amazon. Rondônia State stands out as the largest producer of native fish species in Brazil, with an estimated production of 72,800 tons in 15,810 hectares of water distributed among 4,308 registered and licensed production and commercialization enterprises (MEANTE; DÓRIA, 2017).

In Rondonia, the native fish species with the greatest commercial value are tambaqui (*Colossoma macropomum*), spotted sorubim (*Pseudoplatystoma corruscans*) and pirarucu (*Arapaima gigas*) MEANTE; DÓRIA, 2017). The latter is one of the largest freshwater fish species in the world, as it can reach up to 200 kg, presents carnivorous feeding habits and mandatory aerial breathing, and stands out for its rapid growth and commercial value (SILVA; DUNCAN, 2016). Although pirarucu is important to regional fish farming, information about its nutritional, zootechnical and microbiological aspects is still scarce, especially for its juvenile phase. During this phase, fish experience high mortality rates, are more susceptible to malnutrition due to the lack of live food, have difficulties in food training, and can become infested with endo and ectoparasites; all of which can lead to immunosuppression and favor secondary bacterial infections (SUHET et al. 2011; MEANTE; DÓRIA, 2017).

In this context, few studies have focused on the microbiota present in the water of fish farming tanks/ponds used to cultivate pirarucu. This microbiota depends on several environmental factors, such as physicochemical conditions of the aquatic environment, especially pH, salinity, and degree of eutrophication of the cultivation system (FURNUS et al., 2014). According to Pilarski et al. (2004), the most common fish tank microbiota are *Acinetobacter*, *Aeromonas hydrophila*, *Flavobacterium*, *Moraxella* and *Pseudomonas*. For pirarucu, these microorganisms are opportunistic pathogens that can kill fish grown in high stocking densities (GANDRA et al., 2007; TAVARES-DIAS et al., 2010). The combination of these infectious agents and stress caused by external factors can cause diseases to progress, eventually leading to fish death (BRANDÃO et al., 2006).

Pseudomonas sp. belongs to the Pseudomonadaceae family, and is a Gram-negative bacterium that presents ubiquitous and bacillary morphology. These bacteria are strictly aerobic, mobile with polar flagella and non-sporulating (TORTORA et al. 2005). This bacteria group is composed of *P. chlororaphis*, *P. aeruginosa* and *P. fluorescens*, which stand out as etiological agents of diseases in aquatic species and can even be found in fish from tropical regions.

The species *Pseudomonas* sp. is part of the aquatic ecosystem and is considered a contaminant or invader because it infects a wide variety of aquatic species and adapts to various environmental conditions (PILARSKI et al., 2004; PERESI et al., 2011). It is routinely diagnosed based on isolation and identification of colony morphology, pigment production and biochemical tests (MAIA et al., 2009). This bacteria is commonly isolated in enriched culture media, such as blood agar or selective and different media such as eosin methylene blue agar (EMB) and cetyltrimethylammonium ammonium chloride (cetrimide agar), the latter being specifically used for *P. aeruginosa* due to the microorganism's ability to resist quaternary ammonia compounds (VIANA et al., 2016).

An important characteristic of *P. aeruginosa* is its ability to form biofilm, which makes treating it even more complicated (PERESI et al., 2011). Furthermore, the species is associated with the disease known as "fin rot", which corrodes the affected area and causes high mortality. In many species, contamination causes loss of appetite, hemorrhagic lesions on the skin and at base of fins, petechial hemorrhages in the gills and liver, as well as the accumulation of ascitic fluid in the peritoneal cavity, all of which signify hemorrhagic septicemia and lead to death (FERNANDES et al., 2018).

Microorganisms are present in all environments and adapt to ecological changes, and some play important roles in nutrient cycling in all ecosystems, however, they are generally seen as secondary pathogens that cause diseases during all stages of fish farming, especially initial ones (PILARSKI et al., 2004). Nevertheless, under adequate conditions, controlling the stock density in fish farming and the activities of aerobic organism makes it possible to control water quality through the immobilization of toxic residues, recycling, and increased feed efficiency (TAVARES-DIAS et al., 2010).

Based on the lack of information about bacterial infections in Amazonian fish farming, one of the main requirements for good development of the production chain is information about the biology and health of the fish species used. In this context, this study aimed to report *Pseudomonas* sp. in viscera and dorsum of a juvenile pirarucu *Arapaima gigas* (SCHINZ, 1822), verified through microbiological analysis.

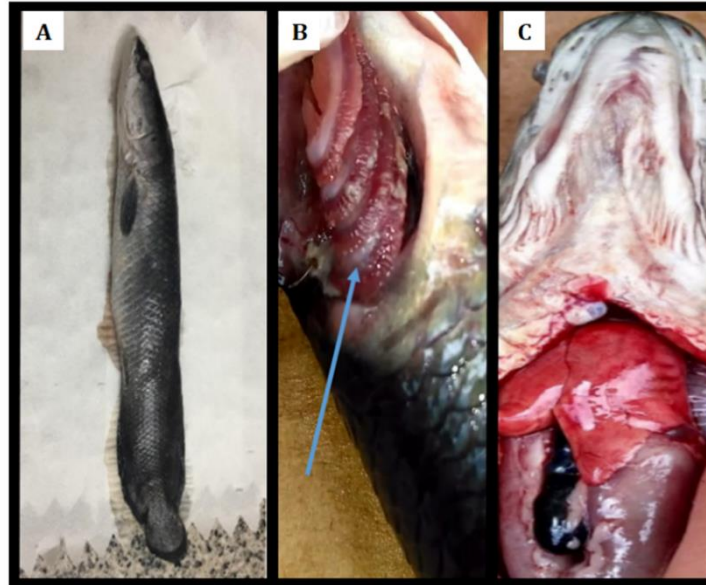
CASE REPORT

This study was carried out through a microbiological analysis of pirarucu (*Arapaima gigas*) samples that were submitted to the Veterinary Microbiology Laboratory at the Sao Lucas University Center, Ji-Paraná Campus, Rondônia. Two 20g-samples of viscera and dorsum were extracted from a pirarucu fish (3 months old and 340g weight). This juvenile pirarucu was collected from a fish farm where the pirarucu fishes presented a history of appetite loss, areas of depigmentation of the skin on

their head and dorsal regions, hemorrhagic spots on their bodies, loss of balance with erratic movements, and

necrotic lesions on their gills and fins (Figure 1).

Figure 1. Juvenile pirarucu (*Arapaima gigas*) submitted to laboratory analysis. (A) whole fish, (B) blue arrow indicates purulent plates on gills and (C) viscera.



The recommendations of Viana et al. (2016) and López et al. (2017) were followed to collect the biological material from the animal. Viscera and dorsum samples were stored separately in sealed sterile plastic bags that were labelled and kept on ice in an isothermal box and sent to the laboratory.

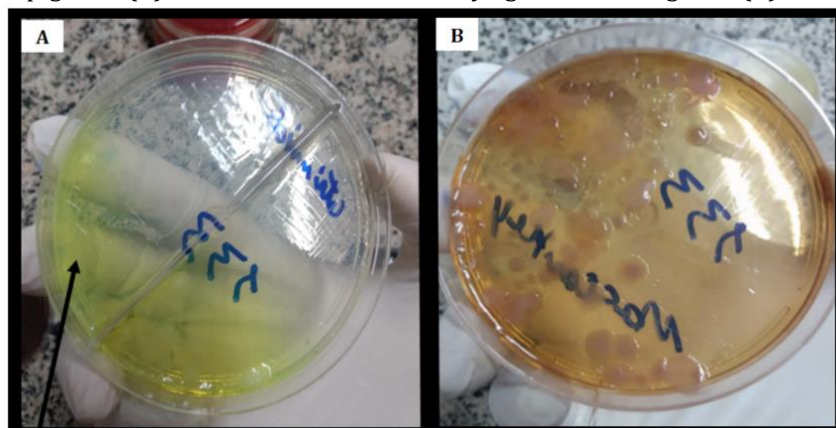
Samples were then segmented and homogenized in a Stomacher-type device along with the Brain Heart Infusion Brothno broth (BHI). The homogenized solution was inoculated on MacConkey agar and Cetrimide agar and incubated in a bacteriological oven at 35 °C for 48 hours. After growth, Gram staining and biochemical tests of catalase, oxidase and Sulfate Indole Motility (SIM) agar, to confirm the diagnosis (MAIA et al., 2009). Bacterial detection was carried out in two main stages, using MacConkey agar, a selective culture medium that promotes the growth of Gram-negative bacteria and inhibits about 98% Gram-positive bacteria due to its bile salts, with the differential of indicating lactose

fermentation (MAIA et al., 2009; BRASIL, 2013). On the other hand, Cetrimide agar selects for *Pseudomonas* sp. because it has cetrimide (cetyltrimethylammonium bromide), which stimulates its growth; colonies were submitted to catalase testing, Gram staining and optical microscope observation (MAIA et al., 2009; BRASIL, 2013).

RESULTS

On the Cetrimide agar, we observed the growth of mucoid colonies and pyoverdine pigmentation (green-fluorescein) (Figure 2A), which, after reacting with pyocyanin (water soluble pigment) resulted in a bright green agglutination, characterized as *Pseudomonas* sp. detection, along with its typical grape (sweet) odor. Its presence was also confirmed by MacConkey agar plating, characterized by the growth of pale colored colonies, mucoid appearance and no lactose fermentation (Figure 2B).

Figure 2. Confirmation of *Pseudomonas* sp. in pirarucu samples (*A. gigas*) using Cetrimide agar. The arrow indicates production of pyoverdine pigment (A) and colonies on MacConkey agar, lactose negative (B).



The presence of the bacteria was confirmed by colony growth in Cetrimide agar, which was submitted to Gram staining and biochemical tests (Catalase, Oxidase, Citrate and SIM medium), with Gram-negative bacilli found through positive oxidase and catalase tests. In the SIM medium, the results were: negative hydrogen sulfide, negative indole and positive motility. Based on Maia (2009), BRASIL (2013) and McVey et al. (2016). From the results, the presence of *Pseudomonas* sp. and *P. aeruginosa* were confirmed in the viscera and dorsum samples from the pirarucu analyzed.

DISCUSSION

In the integumentary system of fish and gills, bacteria such as *Aeromonas hydrophila*, *Alcaligenes piechaudii*, *Enterobacter aerogenes*, *Escherichia coli*, *Flexibacter* spp, *Micrococcus luteus*, *Moraxella* spp, *Pseudomonas fluorescens* and *Vibrio fluvialis* can also be found (BERNARDES et al., 2003). These microorganisms may be present because of various environmental factors and the immune status of fish, including sudden temperature variations, water acidity and pollution of the aquatic environment (GANDRA et al., 2007), as well as inadequate diet, malnutrition, high storage density of fish tanks, irregular transport and mechanical traumas (TAVARES-DIAS et al., 2010).

The pathogenicity of bacteria of the *Pseudomonas* sp. genus in tropical fish is related to the production of certain virulence factors, such as lipopolysaccharides, which cause septic shock syndrome through the release of vasodilation mediators (cytokines such as IL-1 and TNF), as well as activate intravascular coagulation (KURAHASHI et al., 1999). Exotoxin A is a toxin produced by the *Pseudomonas* genus that impairs nerve functions and disrupts the activity of defense cells such as macrophages, while leukocidin is a cytotoxin that inhibits the action of neutrophils and lymphocytes. Pyocyanin is a pigment that inhibits the growth of other bacteria and fimbrias, which are structures that promote adherence to the tissue surface (LÓPEZ et al., 2017; KONEMAN, 2018).

Some species of *Pseudomonas* sp. are considered important pathogens related to urinary tract infections, which can cause chronic infections in the epithelial lining, soft tissue, ophthalmological, dermatitis and folliculitis, as well as other systemic infections (MESAROS et al. 2007). This genus comprises a large number of Gram-negative, non-fermentative, strict and mobile aerobic bacilli since the species present polar flagellum or more. Even though these bacteria are cosmopolitan, some studies, e.g., Fernandes et al. (2018), mention that *Pseudomonas* multiply more rapidly in humid environments, such as watercourses and in the microbiota of aquatic organisms. The main method used to detect such unwanted multiplication is the use of Cetrimide and MacConkey agars (MAIA et al., 2009; FERNANDES et al., 2018).

One way to detect such bacteria is the characteristic and sweet odor similar to green grapes, along with the fluorescence of the bacterium colony by the pyoverdine pigment composition under ultraviolet light (UV). (QUINN et al. 2005). However, Kiska and Gilligan (2003) and Biobrás (2013) emphasize that these findings of growth and biochemical tests are characteristic of the genus *Pseudomonas* sp., nevertheless, pyocyanin pigmentation is exclusively found in *Pseudomonas aeruginosa* since other *Pseudomonas* sp. species do not produce this pigment. Growth on MacConkey agar was possible because the culture medium selects for Gram-negative bacteria, as its crystal violet substance and bile acid inhibit the growth of Gram-positive bacteria. If the bacteria grown in the medium are not lactose fermenters, as in *Pseudomonas* sp., they use agar peptones as a nitrogen source and metabolic products are alkaline, consequently, colonies present a white to greenish color (QUINN et al. 2005).

Bacteriological development in the culture media, along with a negative history of management within fish farming, e.g., decreased control and water supply in the dry season, tank overcrowding, decreased consumption of food, excess feed in water and consequent increased concentration of organic matter, establish conditions that lead fish to situations of stress (LÓPEZ et al., 2017). Similar conditions have been described by Conte (2004) and Brandão et al. (2006), who reported that changes in water quality and management disorders lead to stress, decreasing the production and immunity of individuals. Stress in fish can inhibit their productive performance and affect homeostatic balance. Galhardo and Oliveira (2006) reported that if the environment in which fish live presents stress factors and does not allow them to escape, e.g., circular tanks, significant behavioral changes occur in their swimming rhythms and fight and flight responses, as well as reduce feeding, increase demands for shelter and reduce territorial behaviors.

After external stress, fish present morphological, biochemical and physiological changes called The General Adaptation Syndrome, which is characterized by changes that fish undergo to try to adapt to stress, if avoided, organisms enter into a state of exhaustion and lose their ability to adapt, causing the breakdown of organic homeostasis and making the, susceptible to infectious diseases such as secondary infections caused by opportunistic bacteria like *Pseudomonas* sp. (ROTTA 2003; BRANDÃO et al., 2006). The infection process begins with the alteration or neutralization of the host's natural defenses, which causes immunosuppression (TAVARES-DIAS et al., 2010; KONEMAN, 2018). The pathogenesis of *Pseudomonas* sp. infections is multifactorial, caused by a variety of virulence factors ranging from bacterial adhesion and colonization to systemic spread throughout the body (KURAHASHI et al., 1999; ABU-ELALA et al., 2016). For *Pseudomonas aeruginosa*, pyocyanin is a virulence factor that inhibits the growth of other bacteria and acts as a reducing agent, catalyzing the formation of reactive hydroxyl radicals

and superoxides, which can cause tissue and fimbria damage that promotes its adhesion on surfaces (PEDERSEN, 1992; BRITIGAN, 1993).

Pseudomonas sp. have been detected in several studies, highlighting its occurrence in aquatic environments, especially in freshwater and fish (BERNARDES et al., 2003; BRANDÃO et al., 2006; MAIA et al., 2009; PERESI et al., 2011; BRASIL, 2013; FERNANDES et al., 2018). These studies reported that the disease caused by this bacterium causes hemorrhagic septicemia in fish, which is characterized by the presence of small superficial lesions, local hemorrhages, particularly in gills and operculum, ulcers, abscesses, exophthalmos and abdominal distension. According to Tavares-Dias et al. (2010), internally there can be ascitic fluid accumulation, anemia and lesions in the liver and kidneys. Additionally, the intestines are often devoid of food, which leads to anorexia. These changes have also been described in cases of fish mortality caused by environmental factors associated with bacterial infections (ABU-ELALA et al. 2016).

Fish farms are vulnerable to diseases of bacterial origin due to inadequate water quality monitoring and food management, which increases economic damage caused by high mortality rates (TAVARES-DIAS et al., 2010). In farm systems, there must be a balance between host health, the proliferation of contaminants and the environmental conditions of fish tanks (VAL et al., 2005). Thus, factors that cause physical and physiological stress to fish should be avoided as much as possible, as they favor the spread of infectious diseases (BRANDÃO et al., 2006). Furthermore, it is important to adopt good food management practices, effective water quality control, sanitary certification and routine diagnosis, since these measures are fundamental for preventing the introduction of infectious agents in fish and the mortality of cultivated species (TAVARES-DIAS et al., 2010).

CONCLUSIONS

The effectiveness of the *Pseudomonas* sp. detection method based on specific culture media reinforces the symptomatological findings within fish farming, where more effective control and prevention measures should be adopted. Good sanitary management practices, frequent water quality analysis and balanced nutrition are recommended to maintain a cultivation system suitable for fish, prevent bacterial outbreaks and decrease mortality. Bacteriological analyses of both juvenile and adult pirarucus from fish farms with high mortality rates are recommended, as well as frequent microbiological and physicochemical analyses of the water in farming tanks.

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