Pathogenesis of mixed infection by *Spironucleus* sp. and *Citrobacter freundii* in freshwater angelfish *Pterophyllum scalare*

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1. Introduction

The ornamental fish hobby is among the most popular industries worldwide, creating multi-million dollar industries. The freshwater angelfish *Pterophyllum scalare* is a South American cichlid from Amazonia that is commonly traded in the global ornamental market. The freshwater angelfish and several other native species are commonly farmed in other countries due to their easy reproduction and as a consequence, the export and import become important factors to consider because of the susceptibility of the fish to different pathogens.

Diplomonads from the genus *Spironucleus* (Hexamita) are flagellate protozoa endoparasites that inhabit the fish gastrointestinal tract, but affect the animal systemically by penetrating the intestinal mucosa to reach the blood stream and consequently other organs. This parasite was diagnosed to cause lip tumors in fish and granulomas in the liver and spleen. Infestations of flagellates are among the predominant intestinal diseases in cichlids and are the genera of bacteria notably responsible for causing disease outbreaks. Recent studies, however, have described significant infections by *Citrobacter freundii* in tilapia, carps and eels, which are economically important fish for aquaculture. The infection caused by *C. freundii* results in inflammation and necrotic changes of internal organs, exophthalmia and bleeding in the eyes. *C. freundii* was recently described in freshwater angelfish, however, little is known about this infection compared...(Continued)

...to the literature.
to the other bacteria mentioned. The infections by both of the pathogens discussed in the present study begin inside the gastrointestinal tract. Various studies have noted the importance of innate and adaptive immune cells and their relationship with microorganisms in the gastrointestinal tract of fish [16–18]. More specifically, researches have described the role of mast cells (MC) for different intestinal infections in fish [19]. The present study described in detail the role of MC during chronic infection in a cichlid fish.

In the present study, parasitic and bacterial analyses for the diagnosis of chronic mortality in freshwater angelfish were carried out. For the C. freundii, the pathology was described and the susceptibility to antibiotics was evaluated.

2. Materials and methods

2.1. Fish and necropsy description

Freshwater angelfish were reared in an in-door system. The fish that exhibited chronic mortality (1–3 dead fish per day) were analysed. The fish were kept in 450 L tanks at a density of 120 fish per tank. Water quality parameters were maintained constant for the dissolved oxygen (5.5–6.5 mg/L), pH (6.0–6.5) and temperature (25–27 °C).

All of the necropsied fish (n = 13) were anaesthetized with benzocaine at 0.1 g/L (E1501 Sigma®) and euthanized by severing of the spinal cord for necropsy. All fish presented lethargy, lip tumors, exophthalmia and hemorrhages in the integument (Fig. 1a,b). The fish showed granulomas in the liver and the spleen during the necropsy.

The study followed the Ethical Principles in Animal Research adopted by the National Council for the Control of Animal Experimentation and was approved by the Ethics and Animal Welfare Committee of Universidade Estadual Paulista — UNESP (protocol number 8.102/16).

2.2. Parasitological analysis

The integument and the gills (n = 11) were scraped using glass slides before the fish were anaesthetized. The material was assessed using a Nikon microscope E200® to search for ectoparasites.

To search for endoparasites, fish were necropsied after euthanasia and the contents of the stomach and the intestine were placed on a glass slide. The tissue was scraped using a cover slip and assessed under a light microscope. Slides were stained with May Grünwald-Giemsa-Wright (MGGW) for 10 min to describe the flagella of the parasites.

2.3. Bacterial analysis

The kidney and spleen (n = 2) were collected aseptically, inoculated in TSB broth (Tryptic Soy Broth, himedia®, India) and incubated at 30 °C for 24 h. The samples were then plated on TSA (Tryptic Soy Agar, himedia®, India) at the same temperature and incubation time for gram staining and purity assessment. To identify the isolates, colony PCR was carried out by sequencing the 16S rRNA gene using the primers 8F (5′-AGAGTTTGA-TYMTGGCTCAG-3′) and 907R (5′-CGCTCAATTGTTTATGTT-3′). The PCR conditions were performed as described by Sebastião et al. [20]. The 16S rRNA sequence was identified using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI).

The antimicrobial susceptibility of C. freundii was evaluated using a diffusion method in MHA (Mueller Hinton Agar, himedia®, India) according to Clinical and Laboratory Standards Institute [21]. Briefly, a suspension of each isolate was adjusted to 0.5 McFarland standard turbidity. Using a sterile swab, the suspension was inoculated in MHA agar in duplicate. The antibiotic disks were placed on each plate and were incubated at 28 °C for 24 h. The isolates were classified as resistant or susceptible to each antibiotic depending on the diameter of the inhibition halo, as described for aquatic bacteria [22].

The antibiotic discs used in the study were florfenicol (30 μg, Oxoid®, USA), oxytetracyclin (30 μg, Oxoid®, USA), ampicilin (10 μg, Laborclin®, Brazil), enrofloxacin (5 μg, Sensifar®, Brazil), tetracycline (30 μg, Sensifar®, Brazil), ciprofloxacin (5 μg, Laborclin®, Brazil), cefoxitin (30 μg, Laborclin®, Brazil) and cefalotin (30 μg, Laborclin®, Brazil).

2.4. Histopathology

The kidneys, spleen, liver and intestine from five freshwater angelfish were sampled and fixed in buffered formaldehyde (10%) for the histopathological analyses. Tissue samples were dehydrated in grades of ethanol, cleared in xylene, embedded in paraffin, sectioned at 5 μm and stained with haematoxylin-eosin (HE). Photomicrographs were prepared using the microscope Nikon E200® equipped with a Moticam 2300® system.

3. Results

3.1. Parasitological analysis

No ectoparasites were found in the tegument and the gills. No endoparasites were found in the stomach. The intestinal analysis, however, showed high levels of infection by a microscopic
protozoan with a prevalence of 100%.

The parasites were highly motile, rotating around their own axis (Supplementary video 1). In general, the pyriform shape was noted to have 11.76 ± 0.96 μm in length and 4.32 ± 0.54 μm in width. Six anterior flagella and two additional posterior flagella were observed in the MGGW slides. Based on these characteristics, the parasite was identified as a *Spiroplasma* species.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.micpath.2016.09.002.

### 3.2. Bacterial analysis

Two bacteria (LP15/1 and LP15/3) were isolated from two fish, of which the microbiological analysis revealed rough and opaque colonies (Fig. 2A) formed by gram-negative short rods (Fig. 2B). Both individual colonies were identified as *C. freundii* via colony PCR using the ribosomal 16S gene and direct DNA sequencing, assuming a 100% coverage and identity similar to or higher than 98% of the reference species in GenBank.

The *C. freundii* isolates presented the same pattern of susceptibility to antibiotics (Table 1). The isolates were susceptible only to ciprofloxacin, ceftoxitin and tetracycline. Both isolates were likewise resistant to five out of eight antibiotics tested, including florfenicol and oxytetracycline, which are commonly used in aquaculture.

### 3.3. Histopathology

Histological sections of the liver revealed moderate lipid degeneration (Fig. 3A) and granulomas (Fig. 3B). In the splenic tissue, spherical granulomas (Fig. 3C) were present with a darker core (composed of dead cell material) and surrounded by enzymes, biogenic amines, proteoglycans and proteases.

In the intestine, numerous *Spiroplasma* and intestinal bacteria were observed in the lumen (Fig. 4A). A large amount of MC was observed in the lamina propria (Fig. 4B). The MC were also observed transitioning to the basement membrane (epithelium). This cell was eosinophilic when stained with HE. The granules of the MC ranged in size. Furthermore, free extracellular granules of MC were observed (Fig. 4C).

### 4. Discussion

The freshwater angelfish is one of the most traded species of ornamental fish, however, there is a lack of scientific descriptions of the diseases that affect these animals. Previous studies describe parasitic infestations caused by *Spiroplasma* *vortens* [2,4], *Ichthyophthirius multifiliis*, unidentified trichodinids, *Dactylogyrus* spp., *Gyrodactylus* spp. [23], unidentified Monogenea species [24], *Capillaria* spp [25], and *Tetrahymena* [26], in addition to bacterial infections caused by *Mycobacterium* spp [27,28], and *A. hydrophila* [29].

Hemorrhaging in the eye and exophthalmia were observed in the present study. Similar clinical signs were observed for cyprinids infected with *C. freundii* [14]. These changes are normally associated to bacterial infection, however, *Spiroplasma* affects the fish head and causes lip tumors and bleeding [30], which can extend up to the eyes due to the structures being within close approximation morphologically for this fish species [31], described granulomatous liver, numerous melano-macrophage centers in the spleen and inflammation of the intestine in fish infected by *S. vortens*. Granulomas [32], inflammatory and necrotic changes in the internal organs [14] were also described in fish infected by *C. freundii*. Both *C. freundii* and *Spiroplasma* sp. present similar histopathological alterations in fish, but a unique indicator of spironucleosis is the presence of a lip tumor, which was observed in this study and several other descriptions.

Granulomatous diseases in aquaculture are usually reported in cases of *Mycobacterium* [33] and *Francisella* [34] infections. The histopathology results of the present study, however, indicated that the mixed infection by *Spiroplasma* sp. and *C. freundii* culminated in diffused granulomas of the liver and spleen tissue in freshwater angelfish. A significant and unusual increase in the number of MC was observed in the intestine of infected freshwater angelfish. The involvement of MC in pathogenesis and protective immunity has been shown in some intestinal infections in mammals [35] and fish [19]. The presence of free granules indicates an immune response of MC in infections. Several authors reported that MC, eosinophils and neutrophils have an active form of cell death called extracellular DNA trap (ETosis) that releases free granules [36]. ETosis is a way for these cells to maintain their immunological and inflammatory effects, since the MC granules are provided with lysosomal enzymes, biogenic amines, proteoglycans and proteases.

Bacterial multi-resistance is one of the growing concerns in fish farming worldwide. The present study found 62.5% antimicrobial resistance of *C. freundii*. Similar bacterial multi-resistance was previously reported in ornamental fish [41,42]. Both *C. freundii* isolates showed susceptibility to ciprofloxacin, ceftoxitin and tetracycline, which could be the preferred drugs for treatment. As discussed by Awad [43] and Abd-El-Ghany et al. [7], ciprofloxacin has demonstrated an efficacy worldwide to control or treat bacterial infections in fish. Padua et al. [44] reported a similar result in which *C. freundii* strains isolated from farmed catfish *Pseudoplatystoma reticulatum* were sensitive to ciprofloxacin and tetracycline. Strains of *C. freundii* isolated from ornamental fish from Colombia,
Florida and Singapore, however, were resistant to these antibiotics [42]. Differences in the susceptibility of the same species of bacteria can be justified by the different origins of the strains (environmental change, fish species and previous exposure to antibiotics).

Treatments of parasites are also needed, as parasitosis is shown to be a gateway to the bacteria infection. Dimetridazole, metronidazole, mebendazole [45], garlic and its derivatives [46,47], and other herbs [8] are key molecules with potential to treat spironucleosis. In light of these considerations, the treatment should be performed carefully because the freshwater angelfish is often affected by an anomaly called swim bladder non-inflation during and after treatments [48,49].

The present study reveals diagnostic and pathological descriptions of chronic mortality of freshwater angelfish caused by

### Table 1

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Halo diameter (mm)</th>
<th>Susceptibility testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stain 1 LP15/1</td>
<td>Strain 2 LP15/3</td>
<td>Standard-strain (sensitive)</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>16</td>
<td>15.5</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>23</td>
<td>24.5</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>29.5</td>
<td>29</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>27</td>
<td>26</td>
</tr>
<tr>
<td>Cefalotin</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>20.5</td>
<td>20</td>
</tr>
</tbody>
</table>

R: resistant, S: susceptible.

**Fig. 3.** Histopathology of infected Freshwater angelfish *Pterophyllum scalare*. Lipid degeneration (A) and granuloma (B) in the liver. Splenic granulomas (C) with dark core surrounded by numerous macrophages (white arrow) (D). Scale bars: 100 μm (A), 100 μm (B), 100 μm (C) e 10 μm (D).

**Fig. 4.** Intestinal lumen showing numerous *Spironucleus* (white arrow) around intestinal bacteria (A). Intestinal lamina propria with numerous mast cells (asterisk) (B) with the presence of free granules (white arrow) in the basement membrane attached near a parasite (black arrow) (C). Scale bars: 100 μm (A), 100 μm (B), 10 μm (C).
the mixed infection of *S. vortens* and *C. freundii*. Furthermore, the antibiotics ciprofloxacin, cefoxitin and tetracycline showed potential for future in vivo treatment protocols.

**References**


