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Trypanosomiasis causing mortality outbreak in Nile tilapia intensive farming: Identification and pathological evaluation

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ABSTRACT

Trypanosomes are flagellated parasite protozoans that prey especially on wild fish and have recently been described affecting fish in aquaculture. The present study was carried out during a mortality outbreak of Nile tilapia *Oreochromis niloticus* reared in net cages. Samples of 10 fish in the finishing phase showing unspecific signs, such as anorexia, skin darkening and gill paleness, were analyzed using hemogram, parasitology, microbiology, histopathology, electron microscopy and molecular identification. For microbiological analysis, the kidney was collected aseptically and cultured on tryptone soy agar for 48 h at 30 °C. For parasitological analysis, skin, gills, intestinal contents and blood scrapings were examined. Photomicrographs of the parasite were measured. For molecular identification, blood samples were processed and sequenced for amplification of the 18S rDNA gene. Gills, liver, spleen, kidney, heart and intestine were sampled for histopathological processing. The microbiological results indicated that the fish were not infected with bacteria. Scrapings of the skin and gills revealed the massive presence of kinetoplastids, which were also observed in greater numbers than erythrocytes in the blood. Intestines were not affected by endoparasites. The morphometric characteristics indicated the presence of the *Trypanosoma* genus, which was confirmed in the sequenced samples, where 95% and 98% of the identity were *Trypanosoma* sp. In histopathology, all organs presented different levels of alteration, accompanied by large numbers of the parasite in small and large vessels. The main findings were the description of mast cell infiltrates in the gill and intestine, as well as multifocal aggregates of melanomacrophages in the liver, pancreas, spleen and kidney. Furthermore, the study addresses the newest features of clinical signs of infected fish and possible causes of infestations and compares the diagnosis of this hemoparasite with other hemoflagellates. To our knowledge, this study represents the first outbreak of *Trypanosoma* in Nile tilapia in South America. The authors warn of possible new cases of trypanosomiasis in aquaculture, recommending possible forms of containment and biosecurity measures.

1. Introduction

Information about diseases caused by hemoparasites and their impact on fish health is scarce (Lourenço et al., 2014), especially information related to parasites of the genus *Trypanosoma*. Species of this genus are ubiquitous parasites that can affect both salt and freshwater fish species (Khan, 1977) but mostly in wild environments.

Trypanosoma are described in a large host diversity; they can infect all classes of vertebrates, including reptiles (Marcili et al., 2013), amphibians (Lemos et al., 2013), birds (Svobodová et al., 2015), mammals (Lima et al., 2015) and fish (Kovacevic et al., 2015), and their life cycles involve alternating between host vertebrates and invertebrates, such as annelids (Fermino et al., 2015) and arthropods (Ooi et al., 2015). However, the complete description of the life cycle of *Trypanosoma* is not well understood in aquaculture. What seems to be the consensus among researchers is that leeches are the intermediate host for fish trypanosomiasis and that this parasite enters the host through the suction wound (Negm-Eldin and Davies, 1999; Karlsbakk et al., 2005; Hayes et al., 2014; Corrêa et al., 2016). Within the host, this parasite multiplies and is found in large numbers in the bloodstream of the fish and, consequently, affects all organs.

In South America, reports of trypanosomiasis are common in several armored fish species (Eiras et al., 1989; Eiras et al., 1990; D'Agosto and Serra-Freire, 1993; Fujimoto et al., 2013; Lemos and Souto-Padrón, 2014; Lemos et al., 2015; Molina et al., 2016; Corrêa et al., 2016), *Petenia kraussii* (Grögl et al., 1980), catsharks (Morillas et al., 1987;

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Silva et al., 2005), and in tuvira (Pádua et al., 2011), highlighting that in all these cases, the hosts are wild fish. The most relevant reports of trypanosomiasis in farmed fish are related to groupers in China (Chong, 2005; Su et al., 2014; Wang et al., 2015), and to our knowledge, only Hamid and Babiker (2011) have reported *Trypanosoma* infections in cultured Nile tilapia *Oreochromis niloticus* (Sudan).

Until now, little has been known about the pathologies that strike the hosts of *Trypanosoma* because it is rare to find an infestation of this parasite in such conditions.

Although most of the fish infected by trypanosomiasis are asymptomatic, the high number of parasites in the bloodstream can severely affect the health of the host, causing anemia, leukocytosis, hypoglycemia, splenomegaly (Islam and Woo, 1991; Su et al., 2014) and a massive reduction of thrombocytes (Fink et al., 2015). All this information is relevant and deserves to be investigated, as these cases can culminate in mortalities and serious economic losses in aquaculture.

To provide new insights and impacts on South America aquaculture, this study mainly approaches the clinical signs, diagnosis and pathogenesis of *Trypanosoma* sp. infecting one of the most produced fish species worldwide, the Nile tilapia.

2. Material and methods

The protocol used in this experiment was in accordance with the Brazilian College of Animal Experimentation (COBEA — Colégio Brasileiro de Experimentação Animal) guidelines and was approved by the Ethics, Bioethics and Animal Welfare Committee (CEBEA — Comissão de Ética e Bem Estar Animal) of the FCAV–UNESP–Jaboticabal campus (protocol number 12.383/16).

2.1. Farming conditions and fish

The study was carried out during a mortality outbreak of Nile tilapia *Oreochromis niloticus* farmed in an intensive system (net cages, volume 22.5 m^3, and density of 80 kg m^{-3}) in the Tietê river in the state of São Paulo. Six net cages were affected by the disease and had approximately 50% mortality.

Regarding the history of the property, the producer reported that fish from the six cages came from the same fry supplier and were treated with antibiotics and disinfectants, but the disease was not controlled and mortalities continued to occur.

Ten fish from the finishing phase (average weight 461.2 ± 52.4 g and standard length of 21.8 ± 1.8 cm) showing gill and internal organ pallor, skin darkening, tegument hemorrhage, and aqueous blood were sampled. Except for fish used for research on ectoparasites, all the others were submitted to euthanasia by deepening the anesthetic plane with benzocaine (0.1 g L^{-1}). Samples of blood were collected by puncturing the caudal vessel. Tissue samples from the gills, liver, spleen, kidney, heart and intestine were also collected for histopathology.

2.2. Microbiological analysis

For microbiological analysis, fish were washed in water and disinfected with 70% alcohol, and necropsy was done aseptically in microbiological flow (Bio Seg® 12, Veco, São Paulo, Brazil). The cranial kidney was perforated with a sterile platinum loop that was inoculated on a tryptone soya agar (Oxoid, Hampshire, United Kingdom) and maintained for 48 h in a microbiological incubator (Quimis®, São Paulo, Brazil) adjusted to 30 °C.

2.3. Parasitological analysis: endo and ectoparasites

Skin and gill scrapings were made with glass slides to evaluate the ectoparasites alive. The fresh smears were analyzed under a microscope (Nikon® E200, Tokyo, Japan).

Fish were necropsied for observation of organ alterations. The intestine was opened and the contents spread over glass slides for macro- and microscopic analysis of endoparasites.

2.4. Hematological analysis: hematocrit, blood smears and scanning electron microscopy

Hematocrit (%) was measured using the microhematocrit technique described by Goldenfarb et al. (1971).

Smears were made with total blood, and samples were stained with May Grünwald-Giemsa-Wright (MGGW). The extensions were evaluated under optical microscopy (Nikon® E200, Tokyo, Japan) at 100× magnification.

For scanning electron microscopy (SEM), the total blood of infected animals ($n = 2$) was collected and fixed in 3% glutaraldehyde solution, then buffered with 0.1 M potassium phosphate at pH 7.4 for 48 h. Subsequently, the samples were post-fixed in 1% osmium tetroxide solution for 4 h, washed in the buffer solution and dehydrated gradually in ethyl alcohol. The material was critical-dried (EMS 850, Electron Microscopy Sciences, Pennsylvania, USA), stubs-mounted and impregnated with palladium-gold. Then, the samples were observed and electron-micrographed in a JEOL JSM 5410 (Jeol, Tokyo, Japan) scanning electron microscope operated at 15 kV.

2.5. Morphometric and morphologic analysis of the Trypanosoma

After staining, the photographs of parasites in blood were measured ($n = 50$) with Image-Pro Plus 4.1 software (Media Cybernetics, Silver Springs, Maryland, USA). The following parameters were measured: body length (BL), total length (TL), body width (BW), nucleus length (NL), nucleus width (NW), distance between the anterior portion and the nucleus (AN), distance between the posterior portion and the nucleus (PN), distance between nucleus and kinetoplast (NK), distance between the kinetoplast and anterior portion (KA), and distance between the kinetoplast and posterior portion (KP).

2.6. Metagenomic DNA extraction, PCR and sequencing

Approximately 500 μL of blood was collected ($n = 2$) and immediately stored in liquid nitrogen until DNA extraction, which was performed with the "DNeasy Blood & Tissue" (Qiagen, CA, EUA) kit according to the manufacturer's instructions. PCR was performed using the following specific primers for the 18S rDNA gene of trypanosomes: B (5′ CGAACAACTGCCCTATCAGC 3′) and I (5′ GACTACAATGGTCTC TAATC 3′) generating a fragment of approximately 900 bp (Hamilton, 2003). The PCR reaction was performed using 10 ng of DNA in a reaction containing 1.25 mM $MgCl_2$, 0.2 mM dNTPs, 1 U of Taq DNA polymerase enzyme (Invitrogen), PCR solution buffer (1×) (Invitrogen), and 7 pmol of each primer. The reaction was conducted under the following conditions: initial denaturation at 95 °C for 5 min, 35 cycles of 95 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min and final extension for 10 min at 72 °C. The amplicons were purified with the "MinElute® PCR Purification" kit (Qiagen, CA, USA) and sequenced on the ABI 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA) with the "DNA Sequencing-Big Dye Terminator Cycle Sequencing Ready ABI Prism" kit, Version 3. The sequences obtained were submitted to GenBank (GenBank accession nos. KX866927.1 and KX866929.1) and compared to those already deposited using Megablast.

2.7. Histopathological analysis

Samples from gills, liver, spleen, kidney, heart and intestine were collected ($n = 3$) to describe histopathological alterations. Tissues were preserved in 10% buffered formalin for 24 h and then transferred to different series of alcohol concentrations, cleared in xylene and