ZOOLOGY

Isolation of *Saprolegnia aenigmatica* oomycetes and protocol for experimental infection of pacu (*Piaractus mesopotamicus*)

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ABSTRACT. Successful disease treatment depends on molecular studies under *indoor* conditions with experimental infection protocols that facilitate understanding the disease and the drug`s efficacy. The internal transcribed spacer (ITS) region was sequenced from three isolates, which were identified as *Saprolegnia aenigmatica*. Subsequently, healthy fish were immunosuppressed with dexamethasone (1.2 mg kg^{-1}) and descaled to the skin using a sharp scalpel. These individuals were isolated in individual aquariums maintained at 22°C. Individuals in one group were subcutaneously inoculated with 9,000 zoospores (DDZ treatment), a second group was exposed to oomycetes in water with three colonized baits (DDB), a third group was maintained in water without zoospores (DD), and a control group (C) consisted of healthy animals. After 48 and 96 hours, two animals from each group were euthanized for fungal re-isolation. The fish from groups DD and C did not show clinical signs, and no oomycetes were isolated. The animals from the DDZ and DDB groups showed cotton-wool-like masses on the skin, and *S. aenigmatica* was re-isolated. Thus, for infection using zoospores or baits parasitized by *S. aenigmatica*, an immunosuppressor (dexamethasone) and a sharp scalpel can be used effectively to establish an experimental infection in *P. mesopotamicus.*

Keywords: oomycete; zoosporangium; oogonium; saprolegniasis.

Isolamento do oomiceto *Saprolegnia aenigmatica* e protocolo de infecção experimental em pacu (*Piaractus mesopotamicus*)

RESUMO. O sucesso do tratamento de uma enfermidade depende do estudo de moléculas em condições de laboratório por meio de protocolos de infecção experimental que viabilizam a compreensão da doença e da eficácia dos fármacos. Pela sequência ITS foram identificadas três cepas de *Saprolegnia aenigmatica*. Dessa forma, pacus sadios foram submetidos à imunossupressão com dexametasona (1,2 mg kg^{-1}), esfoliados com auxílio de bisturi e distribuídos em aquários a 22°C. Após este procedimento, um grupo de animais foi inoculado com 9.000 zoósporos/peixe subcutâneo (DEZ), a outro foram adicionadas três iscas colonizadas com o oomiceto na água (DEI), um terceiro grupo foi mantido sem contato com o oomiceto (DE) e um quarto grupo, de animais sadios, representaram o controle (C). Após 48 e 96h deste procedimento, foram eutanaziados animais de cada grupo para reisolamento. Os animais do grupo DE e C não apresentaram sinais clínicos e não foi reisolado o oomiceto. Porém, tanto os animais do grupo DEZ quanto como os animais do grupo DEI apresentaram micélio branco na pele e foi reisolado *Saprolegnia aenigmatica*. Assim, a infecção com zoósporos ou com iscas colonizadas por *S. aenigmatica*, com o uso de dexametasona e abrasivo epitelial são formas eficazes de infecção experimental em *P. mesopotamicus*.

Palavras-chave: oomiceto; zoosporângio; oogônia; saprolegniose.

Introduction

Saprolegnia spp. is an oomycete that causes mycotic diseases in fish and in their eggs in winter (Chukanhom & Hatai, 2004). The fungus generates a cotton-wool-like mass and causes destruction of the skin and fins due to hyphal penetration; the infection can lead to death by hemodilution and osmoregulatory failure (van West, 2006; Fregeneda-Grandes, Rodríguez-Cadenas, & Gancedo, 2007). Globally, *Saprolegnia* is responsible for 10% of the annual economic losses of salmonids (Robertson et al., 2009; van den Berg, Mclaggan, Diéguez-Uribeondo, & van West, 2013).

The genus *Saprolegnia* shows the following characteristics: coenocytic hyphae; mobile zoospores; terminal, subterminal or intercalary zoosporangium; sexual reproduction with different gametangia; a terminal, lateral or intercalary female oogonium; and a male terminal antheridium. In the mycelium, the antheridium grows close to the oogonium and produces an expansion of the fertilization tube. The male nucleus travels through this tube to reach the female nucleus, after which fertilization occurs, and the zygote forms (Ke, Wang, Gu, Li, & Gong, 2009).

The most common species causing disease in fish is *S. parasitica* (Vega-Ramírez et al., 2013), although *S. diclina*, *S. australis*, *S. terrestris*, *S. ferax*, *S. glomerata*, *S. uliginosa*, *S. unispora* and *S. brachydanis* have also caused issues (Diéguez-Uribeondo et al., 2007; Ke et al., 2009; Cao et al., 2012; Vega-Ramírez et al., 2013). Moreover, *S. aenigmatica* and *S. racemosa* were identified recently as being problematic (Sandoval-Sierra & Diéguez-Uribeondo, 2015).

Because a drug study to control *Saprolegnia spp.* infection needs to be conducted, an experiment involving fish infection is essential to determine the efficacy of an antifungal treatment (Howe & Stehly, 1998) and for understanding the disease as well as the resistance capacity of the fish (Singh, Pandey, & Gupta, 2013). An efficacy study requires the isolation, identification and experimental induction of the disease and knowledge of the pathogenicity of the strain.

Authors have used several different approaches for infecting fish with oomycetes to study pathogenicity including cortisol application (Pickering & Duston, 1983), skin epithelial cell damage (Singhal, Jeet, & Davies, 1987), water temperature alteration (Bly, Lawson, Szalai, & Dlem, 1993), xenobiotic exposure (Carballo, Munoz, Cuellar, & Tarazona, 1995), damaged epithelial cells and increased temperature (Howe & Stehly, 1998), and 'ami-momi' treatment (Whisler, 1996; Fregeneda-Grandes, Diez, & Gancedo, 2001; Stueland, Hatai, & Skaar, 2005). In Brazil, no protocols exist for the use of oomycetes to infect native fishes, nor are any drugs registered to control this pathogen in aquaculture.

Therefore, the development of an experimental protocol, especially for use with a neotropical species such as *Piaractus mesopotamicus*, is important for drug development. Accordingly, the aims of this research were to isolate *Saprolegnia* sp. oomycetes using morphological and molecular identification and to develop an experimental infection protocol for *S. aenigmatica* in *P. mesopotamicus* by testing with two types of exposure to the pathogen: zoospores subcutaneously inoculated and fish exposed to zoospores in water.

Material and methods

All procedures performed with animals were in accordance with the ethical standards of the institution at which the studies were performed and were approved by the University's Institutional Animal Care and Use Committee under approval numbers 017335/10.

Collection and isolation of *Saprolegnia aenigmatica*

For the retrieval of isolates, one sample was collected from an *Odontesthes bonariensis* egg at *Universidade Federal de Pelotas* (UFPel) (Rio Grande do Sul State, Brazil) and named PelSS; one sample was collected from tilapia (*Oreochromis niloticus*) fish farm water and named NeSS1; and one sample was collected from tilapia with saprolegniasis (named NeSS2) at the Aquaculture Center of Unesp, Jaboticabal, (São Paulo State, Brazil).

For the water samples, sterile bottles (250 mL) were used for sampling, sesame seeds were added (n = 30) to the sample, and the bottles were kept at 25°C for 48 hours in a photoperiod of 12 hours. The colonized seeds were placed in a Petri dish with potato dextrose agar (PDA) medium and antibiotics (penicillin, tetracycline and chloramphenicol).

Fungus fragments from the eggs and fish samples were removed and used to directly inoculate potato dextrose agar (PDA) medium with antibiotics (penicillin, tetracycline and chloramphenicol) in a Petri dish.

After growth of the fungus (white mycelia with a cotton-like appearance), the samples were separated for morphological and molecular identification and zoosporogenesis.

Morphological identification

A disk of oomycetes (6 mm) was placed in the center of a PDA plate, and baits (sesame seeds and pieces of *Stenotaphrum secundatum*) were distributed around it. The disk was kept at 25°C for 24 hours. The colonized seeds were placed in a dish with sterile, distilled water and kept at 25°C for 48 hours. Subsequently, the baits were observed under a light microscope (Labomed LX400) to verify identification by the hyphae, zoosporangium, zoospores and sexual structures according to Sanvodal-Sierra and Diéguez-Uribeondo (2015).

Sequencing of the ITS region and analysis

Isolates were cultivated for seven days in potato dextrose liquid medium to allow separation of the

fungal mycelia. The mycelia were dried and ground in liquid nitrogen, and DNA was extracted according to the protocol of Kuramae-Izioka (1997), with modifications.

The ITS region was amplified using the primer pair ITS1/ITS4 (White, Bruns, Lee, & Taylor, 1990). The PCR reaction consisted of 1x Buffer (20 mm Tris-HCl pH 8.4; 50 mm KCl), 2 mm MgCl$_2$, 0.2 mm dNTPs, 1.0 U Taq DNA polymerase, 5 pmol each primer, 60 ng genomic DNA and water to 20 μL volume. Amplification was carried out in a thermocycler, as follows: one cycle at 95°C for 4 min; 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 60 s; and one cycle at 72°C for 10 min. The amplified products were analyzed by electrophoresis in 1.0% agarose gels (w v^{-1}).

The amplicons were partially sequenced in the forward and reverse directions using the same primers used for amplification. The sequencing reaction was performed using a BigDye Terminator v3.1 Kit (Applied Biosystems), and capillary electrophoresis was carried out using an ABI3100 sequencer (Applied Biosystems). The Phred/Phrap/Consed software package was used to verify base quality and to assemble and trim consensus sequences (Gordon, Abajian, & Green, 1998), which were then compared with sequences in GenBank. *Saprolegnia* reference sequences (Sandoval-Sierra, Martín, & Diéguez-Uribeondo, 2014; Sanvodal-Sierra & Diéguez-Uribeondo, 2015) and sequences from *Achlya debaryana* and *Aphanomyces astaci,* used as the outgroups (Diéguez-Uribeondo et al., 2007), from GenBank were aligned with those obtained in this study using MUSCLE version 3.6 with default parameters (Edgar, 2004). The substitution model was selected using the Akaike information criterion (AIC) in MEGA 6.06 software (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Bayesian analysis was performed using MrBayes 3.2.3 software (Ronquist & Huelsenbeck, 2003) with four independent Markov chain Monte Carlo (MCMC) runs of one million generations, with sampling every 200 generations. The initial 25% of the sampled trees were discarded as burn-in.

Experimental protocol for *Saprolegnia aenigmatica* infection of *Piaractus mesopotamicus*

The strain used for inoculation was the NeSS1 isolate from water. Zoospores were quantified in a Neubauer hemocytometer with a light microscope (400x) (Zahran & Risha, 2013). The quantity per mL was estimated, and the inoculation volume was

defined (National Committee for Clinical Laboratory Standards [NCCLS], 2008).

For experimental infection, healthy fish (average weight 60 ± 5.2 g) were anesthetized (benzocaine 0.1 g L^{-1}) and immunosuppressed with 1.2 mg kg^{-1} dexamethasone (Claudiano et al., 2013). Four fish were descaled with a sharp scalpel and inoculated subcutaneously with 9,000 zoospores for the first treatment (dexamethasone, descaled, zoospores: DDZ treatment). Another four animals that had been immunosuppressed and descaled were subsequently exposed to oomycetes in the water using four colonized baits (sesame seeds) (dexamethasone, descaled, baits: DDB). Four more animals that had been immunosuppressed and descaled were not exposed to oomycetes (dexamethasone, descaled: DD). Four healthy animals were used as the control (C). Because this protocol for experimental infection with zoospores is new, the conditions of the test should be monitored constantly; thus, a smaller number of fish can be evaluated better during the infection process.

The treatments with healthy fish that had been descaled and exposed to parasitized seeds and with healthy fish immunosuppressed (dexamethasone) and exposed to parasitized seeds parasitized had been studied previously; these treatments showed no clinical signs (mycelium), and the pathogen was also not re-isolated. Therefore, these treatments are not included in the protocol.

The fish were kept in individual aquariums with 6 L of water, artificial oxygen, a 12 hour photoperiod and a temperature of 20.0 ± 2.0°C. The water was maintained as follows: pH 8.0, dissolved oxygen > 6.0 mg L^{-1}, electric conductivity between 170.0 and 180.0 μS cm^{-1}.

Each assay had four replicates, and the animals were fed daily. The clinical signs were evaluated daily, for the presence of mycelium. After 48 and 96 hours, two animals from each treatment were collected for the re-isolation of *S. aenigmatica*. Pieces of skin were removed, placed on PDA medium with antibiotics (penicillin, tetracycline and chloramphenicol), and incubated for 72 hours at 25°C. If the colonies were suggestive of *Saprolegnia,* zoosporogenesis was induced to analyze the reproductive structures and to verify the species.

Results and discussion

Morphological features

The oomycete produced voluminous mycelia on PDA medium (cotton-wool-like appearance). When

visualized under the microscope, the hyphae were coenocytic, long and wide.

The parasitized baits exhibited a white mycelium with terminal zoosporangium with a brown color. Many zoospores were in the latent or mature stage; the zoosporangia may have been empty because the zoospores had been released, or secondary zoosporangia could have been generating on the baits. The oogonium was terminal and in an initial stage or mature with oospores, which were circular cells with a dark cytoplasm; some exhibited antheridial cells. The zoospores originating from asexual reproduction had thin membranes and clear color, and the zygote resulting from sexual reproduction was a larger cell and had a dark color and a thick membrane (Figure 1).

Molecular characterization

The sequences obtained from egg, water (PelSS and NeSS1) and tilapia (NeSS2) were 100% similar to the sequences of *S. aenigmatica* deposited in GenBank (Figure 2).

### Experimental protocol of *Saprolegnia aenigmatica* infection of *Piaractus mesopotamicus*

The control and DD groups did not show any clinical signs, and no oomycetes were found among the samples collected.

However, the animals from the DDZ group, in contrast to the DDB group, showed clinical signs at 48 hours after infection, including grayish, cotton-wool-like, white mycelia on the skin and in the fins, mouth and eyes after 72 hours. The DDZ group showed more advanced signs with greater mycelial masses.

The clinical signs remained for up to 96 hours; some animals were moribund due to opportunistic bacteria and were euthanized (a high dose of anesthetic) for fungal re-isolation. Clinical signs in animals that did not display very obvious clinical symptoms (few mycelia) began to decrease.
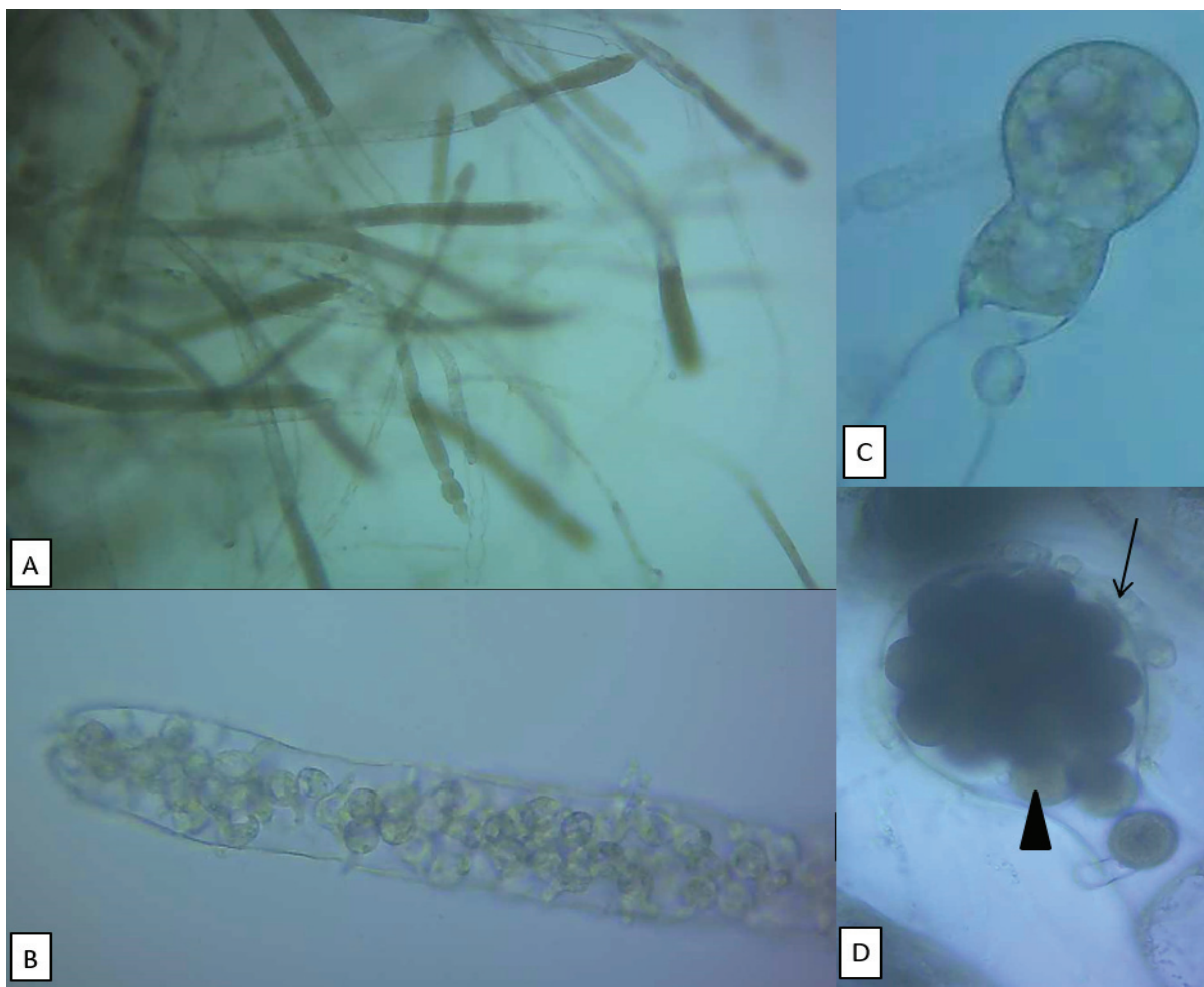
Figure 1. A. Zoosporangia at different stages. B. Zoosporangium with zoospores (40X). C. The oogonium (40X). D. An oogonium with oospheres (head arrow) and antheridial cells (arrow) (40X).
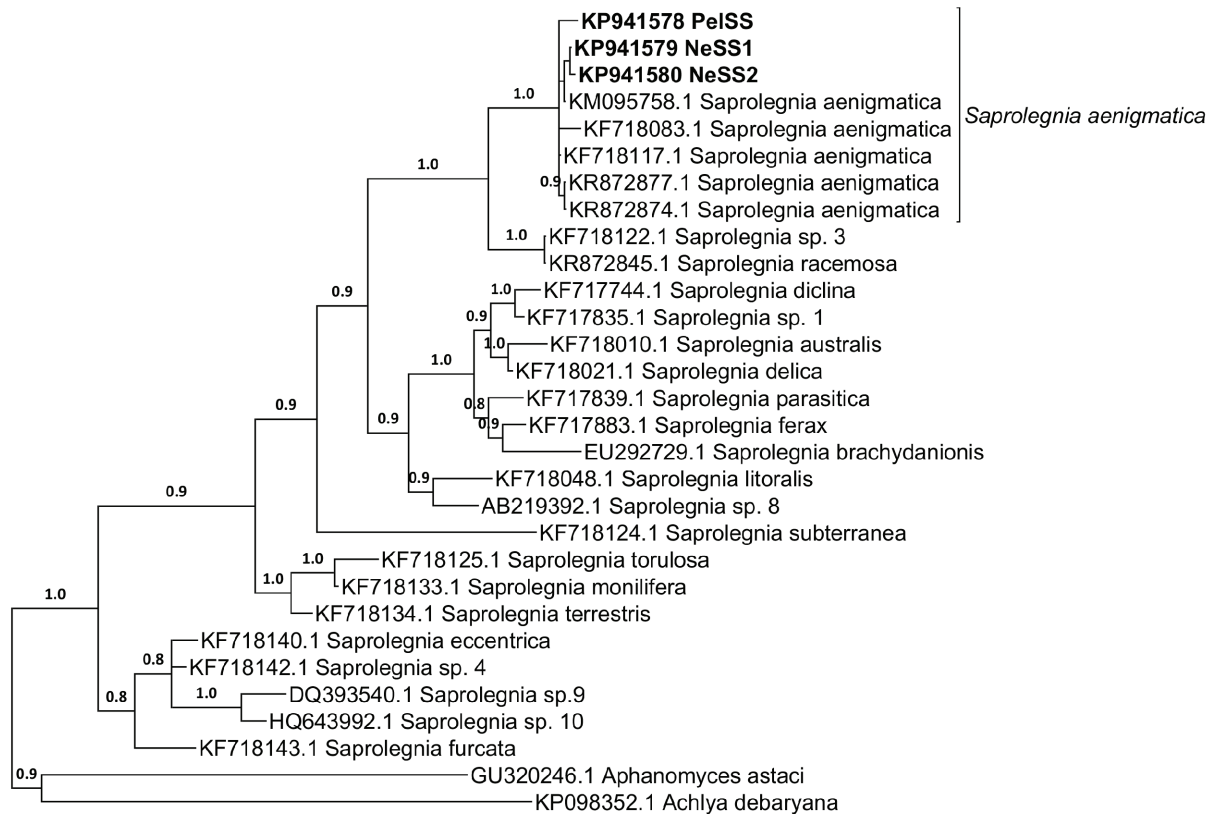
Figure 2. Bayesian analysis of the ITS rDNA sequences from *Saprolegnia* isolates from fish with clinical signs of saprolegniasis and from fish farm water. The numbers in the branches are related to probability values, with a standard deviation of < 0.01, from Bayesian analysis.

Samples from the DDZ and DDB groups collected at 48 and 96 hours revealed that the mycelia, hyphae, zoosporangia and zoospores produced were those of *S. aenigmatica* (Figure 1). This finding suggested that the fish of both groups were initially inoculated with this pathogen. Although the DDB group showed clinical signs only 72 hours after inoculation, *Saprolegnia* was isolated at 48 hours after inoculation.

These results showed that 100% of the fish exposed to zoospores of *Saprolegnia,* whether inoculated in the skin or from parasitized seeds, were infected.

Oomycete isolation can be performed using fish, eggs, water and soil. In this study, *S. aenigmatica* was isolated from water, eggs and fish. Céspedes and Castillo (1982) isolated *Aphanomyces sp.*, *Leptolegnia* sp. and *Saprolegnia sp.* from the ground and from water, Vega-Ramírez et al. (2013) isolated eight species of *Saprolegnia* from water, eggs and trout with lesions, and Corrêa, Stohl, Robaldo, and Pereira (2013) isolated six strains of *Saprolegnia sp.* from water and 12 strains from eggs.

The morphological features used in this study of *S. aenigmatica*, such as the oogonium, antheridium, zoospore, zygote and hyphae, are commonly used for oomycete identification (Zaror et al., 2004; Shah, 2010; Das et al., 2012; Vegas-Ramírez et al., 2013; Ali et al., 2013; Corrêa et al., 2013).

The most common species causing disease in fish is *S. parasitica* (Zaror et al., 2004; Stueland et al., 2005; Diéguez-Uribeondo et al., 2007; Ali et al., 2013; Vega-Ramírez et al., 2013). In addition, *S. diclina*, *S. australis*, *S. terrestris*, *S. ferax*, *S. glomerata*, *S. uliginosa*, *S. unispora* and *S. brachydanis* have caused issues (Diéguez-Uribeondo et al., 2007; Ke et al., 2009; Fuangsawat, Abking, & Lawhavinit, 2011; Cao et al., 2012), and *S. aenigmatica* and *S. racemosa* were also recently reported as being problematic (Sandoval-Sierra & Diéguez-Uribeondo, 2015). However, no record is available regarding the sensitivity of these different species to drugs.

In our experimental protocol, clinical signs were visible at 48 hours after infection. Fregeneda-Grandes et al. (2001) also observed the first clinical signs in the skin, eyes, nostrils and operculum of *O. mykiss* within 48 hours.

The same clinical symptoms were also observed in *O. mykiss* infected with *S. parasitica* (Whisler,

1996), *S. salar* infected with zoospores of *Saprolegnia* sp. (Stueland et al., 2005), and *O. niloticus* infected with *Saprolegnia* sp. (Zahran & Risha, 2013). In addition, necropsy revealed skin and fin destruction, yellow mucus in the gut and hemorrhagic enteritis (Fregeneda-Grandes et al., 2001).

The clinical signs remained for up to 96 hours; some animals were moribund due to opportunistic bacteria. In some animals that did not have obvious clinical symptoms (few mycelia in the skin) and others, the signs began to decrease. Whisler (1996) described animals with milder clinical signs as tending to exhibit decreased symptoms after one week, reporting that the animals that died showed clinical signs of bacteriosis.

A pathogenicity study was also performed using *S. ferax* zoospores in water (2×10^4 zoospores liter^{-1}) with tilapia previously descaled in different regions on the skin (Zahran & Risha, 2013) and according to Roberts (2012), epithelial damage to the skin, gills and gut could provide a route for oomycete entry.

Dexamethasone, a glucocorticoid that inhibits vasodilation, edema, chemotaxis and phagocytosis (Ryan, Griffin, & Connor, 2011), has been used in fish studies (Claudiano et al., 2013). In this research, dexamethasone was used with descaling to facilitate *S. aenigmatica* infection.

Some techniques have been used to study the oomycete pathogenicity in some fish species such as *Salmo trutta* (Pickering & Duston, 1983), *Cyprinus carpio* and *Labeo rohita* (Singhal et al., 1987), *Ictalurus punctatus* (Bly et al., 1993), *Oncorhynchus mykiss* (Carballo et al., 1995; Whisler 1996; Fregeneda-Grandes et al., 2001) and *Salmo salar* (Howe & Stehly, 1998; Stueland et al., 2005). However, no similar study has been conducted in Brazilian native fishes, and for the protocol described in this research, 100% of the *P. mesopotamicus* were infected, showing either severe signs or a few mycelia.

Whisler (1996) used seeds parasitized with *Saprolegnia* and found clinical signs in *O. mykiss* eight days after zoospore exposure. *Piaractus mesopotamicus* exposed to *S. aenigmatica* manifested clinical signs in 48 and 72 hours after exposure, showing that this strain had high pathogenicity for this fish.

Saprolegnia aenigmatica was recently identified by Sandoval-Sierra and Diéguez-Uribeondo (2015) and according to them, more than 60% of *S. aenigmatica* isolates are sterile; that is, they do not form sexual structures, and the features observed are not species specific. However, the three strains in this research (PelSS; NeSS1 and NeSS2) produced sexual structures, and all shared 100% similarity with *S. aenigmatica*.

The present experiment accomplished the four steps of Koch's postulates for pathogens (Koch, 1932): (1) the pathogen must be present in all cases of the disease and not in healthy animals; (2) the pathogen can be isolated from a diseased host and grown in pure culture; (3) the pathogen from pure culture must cause the disease when inoculated into a healthy, susceptible laboratory animal; and (4) the pathogen must be re-isolated from the new host and shown to be the same as the originally inoculated pathogen. Few studies exist on the impact of *Saprolegnia* in aquaculture, and this research contributes to the study of oomycetes, their identification and pathogenicity for a native fish, *P. mesopotamicus*. In addition, this study contributes to future analyses of drugs used to treat such diseases. Accordingly, it is clearly necessary to study the oomycetes that cause disease in fish during production.

Conclusion

Three strains of *Saprolegnia aenigmatica* were isolated and identified according to their morphological and molecular features. Experimental infection with zoospores or baits parasitized by *S. aenigmatica*, in conjunction with an immunosuppressor (dexamethasone) and descaling, was an effective way to achieve experimental infection in *P. mesopotamicus*.

Acknowledgements

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