



NOTE

Pathology

Candida parapsilosis and *Candida tropicalis* infections in an Okhotsk snailfish (*Liparis ochotensis*)

Mohie HARIDY^{1)*}, Walied ABDO²⁾, Mahmoud HASHEM³⁾ and Tokuma YANAI^{4)*}¹⁾Department of Pathology & Clinical Pathology, Faculty of Veterinary Medicine, South Valley University, Qena 83523, Egypt²⁾Department of Pathology, Faculty of Veterinary Medicine, Kafr-Elsheikh University, Kafr-Elsheikh 33516, Egypt³⁾Department of Fish Diseases and Mangment, Faculty of Veterinary Medicine, New Valley Branch, Assuit University, El-Kharja 72511, Egypt⁴⁾Department of Pathogenetic Veterinary Science, United Graduated School of Veterinary Science, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan*J. Vet. Med. Sci.*

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ABSTRACT. An Okhotsk snailfish (*Liparis ochotensis*) kept at Nagoya aquarium exhibited sudden death. Microscopically, the fish showed multiple granulomatous foci in the gills, liver and kidney. Multiple yeast-like organisms as well as pseudohyphal elements were observed within granulomatous lesions. Immunohistochemically, the hyphae were negative for both *Aspergillus* and *Mucor* spp., and a weak positive for *Candida* sp. The seminested-PCR product was consistent with *Candida parapsilosis* and *C. tropicalis*. This is the first record of disseminated mycotic granulomatous lesion due to *C. parapsilosis* and *C. tropicalis* infection in fish.

KEY WORDS: *Candida parapsilosis* and *C. tropicalis*, mycosis, Okhotsk snailfish

Fungi may be primary or secondary pathogens [18]. *Candida* species are dimorphic fungi in the family *Saccharomycetaceae*. In the yeast phase, it normally inhabits the mucosal alimentary, upper respiratory and genital tracts of human and animals [20]. *Candida parapsilosis* (*C. parapsilosis*) is ubiquitous commensal organism of a variety of species and even be isolated from soil [20]. *Candida tropicalis* (*C. tropicalis*) was widely distributed in tropical and subtropical marine environments [25]. Also, it is isolated from expired breathes of captive dolphins and their environment, and sometimes circulate the pathogenic yeast between them [22]. *C. parapsilosis* and *C. tropicalis* are considered the most prevalent infections of non-*Candida albicans*-mycosis. It usually requires an immunocompromised host. *C. parapsilosis* have been associated with disease in human such as endocarditis, meningitis, septicemia, peritonitis, arthritis, endophthalmitis, keratitis, otitis, cystitis, and skin infections [20, 23]. Interestingly, *C. parapsilosis* has been isolated from cats and cockatiels after a history of eating marine foods [11, 15, 19]. *C. tropicalis* have increased dramatically on a global scale and considered to be emerging pathogenic yeast, and involved with candidemia and invasive candidiasis [14]. The present study aimed to diagnose the causative agent of mycosis in an Okhotsk snailfish (*Liparis ochotensis*) using seminested PCR (sn-PCR) technique on formalin-fixed paraffin embedded (FFPE) tissue.

An Okhotsk snailfish kept in the Port of Nagoya public aquarium, exhibited sudden death, then were submitted for necropsy to laboratory of Veterinary Pathology, Gifu University. Samples from the gills, liver, kidney and different organs were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections (3 μ m) were stained with hematoxylin and eosin (H&E), periodic acid Schiff (PAS) and Grocott's methenamine silver (GMS). PAS and GMS satins were used to investigate the distribution of yeast in different tissues i.e. intra- /extra-vascular or both.

Immunohistochemistry was carried out using anti-*Aspergillus*, Anti-*Rhizomucor* and anti-*Candida albicans* antibodies. Mouse anti-*Aspergillus* spp. monoclonal antibody (WF-AF-1; AbD Serotec, Oxford, U.K.), mouse anti-*Rhizopus arrhizus* monoclonal antibody (WSSA-RA-1; AbD Serotec) and rabbit anti-*C. albicans* type A polyclonal antibody (AbD Serotec) were used in a suitable dilution as follow 1:200, 1:100 and 1:200, respectively [18]. Paraffin-embedded sections of gills and liver of the fish were investigated for detection of fungal antigen using two-step polymer method (Dako EnVision™). Briefly, sections were deparaffinized in xylene and rehydrated in graded ethanol. Antigen retrieval pretreatment was carried out by heating the tissue sections in target retrieval solutions (pH 6.0) (DakoCytomation, Glostrup, Denmark) in an autoclave for 10 min at 105°C. Endogenous peroxidase was quenched with 0.3% hydrogen peroxide in methanol for 20 min at room temperature (~28°C). To prevent the binding of nonspecific proteins to the primary antibodies, the sections were treated with Protein Block Serum-free

*Correspondence to: Yanai, T.: yanai@gifu-u.ac.jp, Haridy, M.: mohieharidy@svu.edu.eg

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Table 1. Primers used in seminested PCR for amplification of *Candida* sp.

Primer	Sequence
CTSF	5'-TCGCATCGATGAAGAACGCAGC-3'
CTSR	TCTTTTCCTCCGCTTATTGATATGC
CADET	ATTGCTTGCGGCGGTAACGTCC
CPDET	ACAAACTCCAAAACCTTCTTCCA
CTDET	AACGCTTATTTTGCTAGTGGCC
CGDET	TAGGTTTACCAACTCGGTGTT

(DakoCytomation) for 30 min. Primary antisera were applied then incubated overnight at 4°C. The sections were then incubated with the anti-rabbit and anti-mouse secondary antibodies (EnVision+™ System HRP Labelled Polymer) for 30 min at room temperature. The sections were rinsed with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO, U.S.A.) between each step. After signal detection by using a freshly prepared solution of 3, 3'-diaminobenzidine tetrahydrochloride (Liquid DAB + Substrate–Chromogen System; DakoCytomation), the sections were washed in distilled water, counterstained with Mayer's hematoxylin, and dehydrated. For negative control, the primary antibody was replaced with PBS using section of non-infected fishes.

Seminested PCR: Molecular diagnosis using sn-PCR assay was performed as previously described. [2]. Universal primers for *Candida* sp. and specific primers for *C. albicans*, *C. parapsilosis*, *C. tropicalis* and *C. glabrata* were used (Table 1). CTSF and CTSR are for amplifying the 3' end of 5.8S rDNA and the 5' end of 28S rDNA of *Candida* sp. Species-specific primers were derived from the ITS2 regions of *C. albicans* (CADET), *C. parapsilosis* (CPDET), *C. tropicalis* (CTDET) and *C. glabrata* (CGDET).

DNA was extracted from FFPE tissues, according to the manufacturer's protocol, using the DEXPATH kit (Takara Bio Inc., Kusatsu, Japan). Briefly, xylene was used to deparaffinize the sections (5 µm thick) prepared from fish tissues, and the resulting tissue pellets were washed with 1 ml 100% ethanol. Residual ethanol was allowed to evaporate by leaving the microcentrifuge tubes open at room temperature for 30 min. The pellets were resuspended in lysis buffer (180 µl) and proteinase K (20 µl), mixed by vortexing, and incubated overnight at 56°C to allow complete digestion of the tissues. The lysed tissue was loaded onto the elution column and washed twice with buffer; the DNA was eluted off the column using 40 µl elution buffer then stored at -20°C until use [10].

Amplification of target DNA was carried out as described by Ahmad *et al.* [2] using thin-walled 0.2 ml PCR tubes and TakaRa PCR amplification kit (Takara Bio Inc.). A total volume of 50 µl PCR reaction contained 5 µl Taq PCR buffer, 0.25 U of Taq™ DNA polymerase, 10 pmol (1 µl) each of CTSF and CTSR primers, 2 µl of DNA extracted from tissue, and 0.1 mM each dNTP. After amplification in the first step, 1 µl of the product was further amplified using the initial reverse primer (CTS1R) and a species-specific forward primer in four separate tubes corresponding to each of the *Candida species* to be detected. For sn-PCR, The reaction mixture consisted of 1 × Taq PCR buffer I; 1 U of Taq DNA polymerase; 5 pmol of CTSR together with 5 pmol of CADET, CPDET, CGDET, or CTDET; 1 µl of the first PCR product; and 0.1 mM each dNTP. Except primers, all reagents were from Takara Bio Inc. PCR cycling was performed in a TakaRa Thermal Cycler (Takara Bio Inc.) in the following conditions: denaturation at 94°C for 1 min, annealing at 60°C for 30 sec, and extension at 72°C for 1 min. An initial denaturation step at 94°C for 3 min and a final extension step at 72°C for 10 min also included. The optimum amplification was obtained with 35 cycles of the first PCR followed by 25 cycles of the sn-PCR. Amplified DNA fragments were detected using agarose gel electrophoresis. The gels were exposed to UV light and photographed. The sizes of amplified DNA fragments were identified by comparison with molecular size marker DNA (100-bp DNA ladder).

Histopathologically, disseminated granulomatous lesion was observed in gill (a, b), liver (c, d) and kidney. Granulomatous lesion in gills is characterized by extensive necrosis rich with heterophils, macrophages and fibroblasts involving the cartilage of gill arches and extending to gill filaments. Several gill filaments were exfoliated due to necrosis of the cartilaginous articulation in gill arch. Blood vessels of the gill arch were thrombosed with pseudohyphal invasion (Fig. 1a). Blood vessels were occupied by blood constituents, hyphae, macrophages and lymphocytes and surrounded by fibroblastic proliferation and mononuclear cell infiltration. Many of pseudohyphae were seen to invade the blood sinuses of the primary lamellae and cartilaginous rod of the primary lamellae (Fig. 1a inset). Apart from the massive ischemic necrotic areas, dispersed secondary lamellae were necrotic and fused together, and many of yeast-like organisms were noticed among the necrotic epithelial cells (Fig. 1b). Thrombosis of blood vessels in affected lamellae was not noticed. Hepatic tissue showed multiple granulomas characterized by center areas of necrosis surrounded by heterophil, lymphocyte, macrophage and fibroblast infiltration. Pseudohyphal elements and yeast-like organism were observed in the granulomatous lesion as well as within the surrounding hepatocytes (Fig. 1c). Most of necrotic areas located perivascular, with intense pseudohyphal elements thrombosis (Fig. 1d). Also, similar lesions were noticed in renal tissues with thrombosis of the renal blood vessels with pseudohyphal invasions (Fig. 1e). By PAS and GMS stains, long pseudohyphae and blastoconidia arised singly or in small groups along the pseudohyphae (Fig. 1b–f). Immunohistochemistry, the reaction using anti-*Aspergillus*, anti-*Rhizopus arrhizus* and anti-*C. albicans* antibodies revealed negative staining except for mild positivity for *C. albicans*.

The PCR amplification of rDNAs from the four *Candida* species, viz., *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C.*

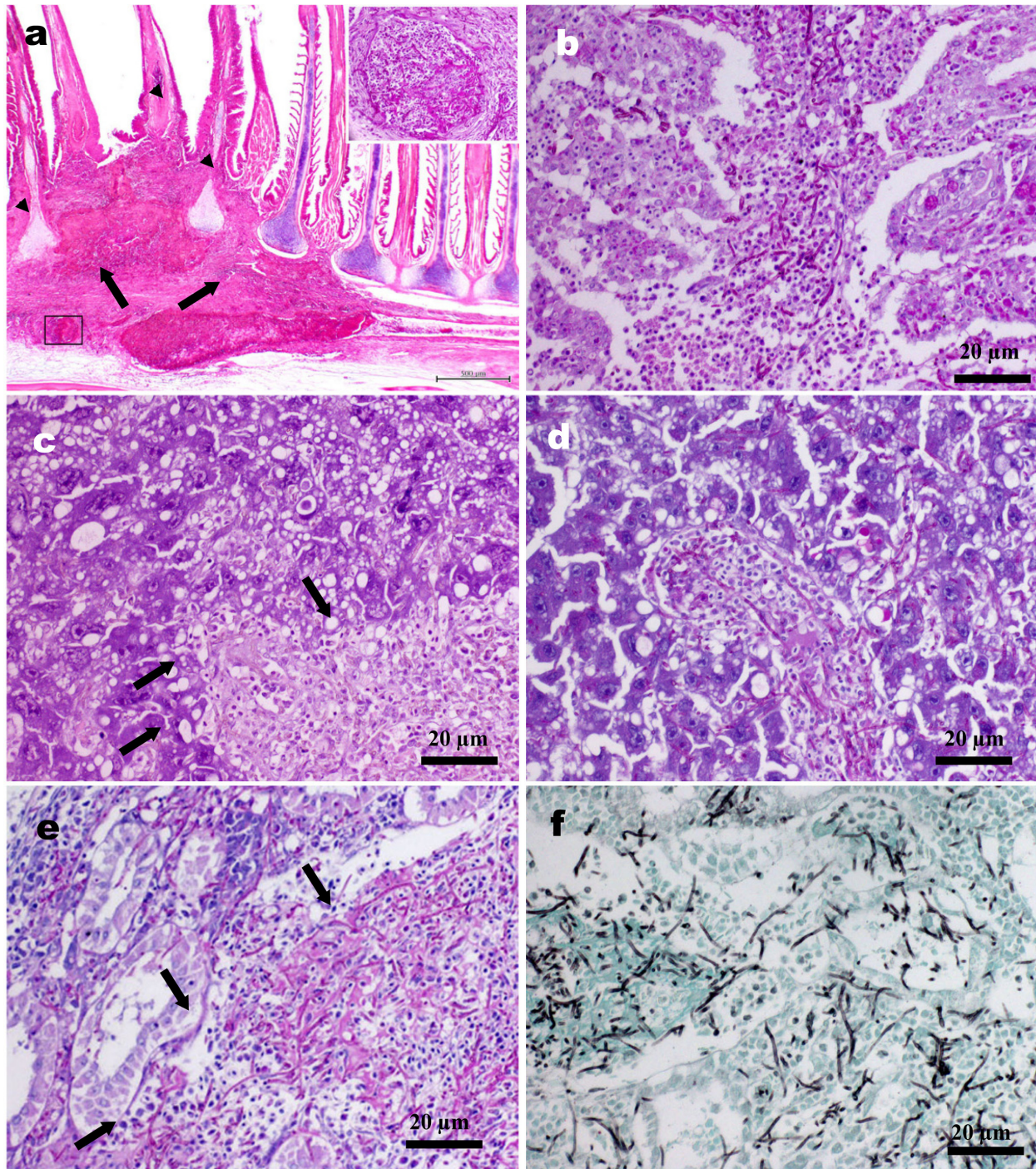


Fig. 1. Systemic mycosis in Okhotsk snailfish characterized by disseminated granulomatous lesion in gill (a, b), liver (c, d) and kidney (e, f). The gill arch revealed extensive necrosis (arrow) (a) with vascular thrombosis (a, inset) and necrosis of the cartilaginous rod (arrowheads) and exfoliation of the primary lamellae (H & E, bar=500 μ m). Secondary lamellae were necrotic and fused together, and many of yeast-like organisms were noticed among the necrotic epithelial cells (b). Granulomas in hepatic (c) and renal (e) tissues (arrows) composed of pseudohyphae, lymphocytes, macrophages and blood cells. Long pseudohyphae and blastoconidia arised singly or in small groups, that invade surrounding blood vessels (d, f); (b–e, PAS stain, bar=20 μ m; f, GMS stain, bar=20 μ m).

glabrata, using universal fungal primers (CTSF and CTSR) resulted in amplification of a single DNA fragment of the expected size (~500 bp) (Fig. 2a). Reamplification of the product of the first PCR with CTSR and the species-specific primers corresponding to the ITS2 sequences from *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata* resulted in specific amplification of single DNA products of the expected sizes (Fig. 2b). CTSR / CPDET and CTSR / CTDET universal primer sets for *C. parapsilosis* and *C. tropicalis*, respectively amplified a ~100-bp and ~140-bp product in sn-PCR, respectively. Use of universal primer for *C. albicans*

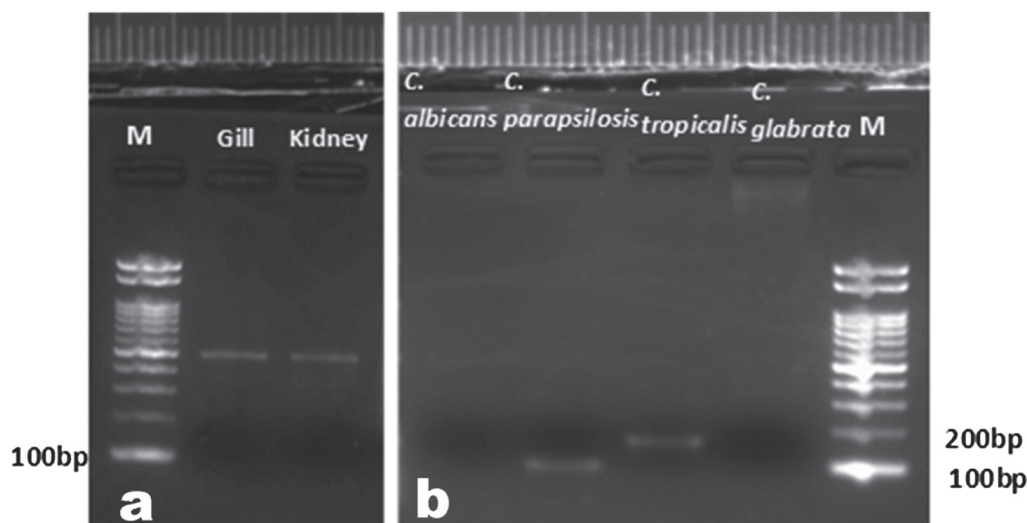


Fig. 2. (a) PCR amplification of genomic DNAs of *Candida* sp. with universal fungal primers. (b) Seminested PCR amplification using the primer CTSR with primers (CADET, CPDET, CTDET and CGDET) specific for *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata*, respectively. Lane M, 100-bp molecular size marker.

and *C. glabrata*, specific DNA was failed to be amplified (Fig. 2b).

Okhotsk snailfish (*Liparis ochotensis*) is a ray-finned fish that belongs to family *Liparidae*. It is wide spread in Sea of Japan, Sea of Okhotsk, pacific of Hokkaido islands and northern and eastern of Bering Sea. Captivity represents the supreme stressful condition, and usually associated with opportunistic fungosis. Fungi in marine mammals and fish portrayed with long-term chronic debilitation and eventual death [1, 21]. Although the water of the aquarium as well as the accompanying fish had not any abnormal clinical signs at time of current case examination, sporadic cases under captivity are usually affected.

Candida albicans, an opportunistic yeast-like pathogen, inhabits the digestive tract and mucosal regions of mammals and birds, and is occasionally isolated from aquatic habitats receiving city sewages, indicating fresh fecal pollution [5]. Recently, *C. albicans* is isolated from multiple skin ulcers in both Nile tilapia juveniles and Sharp toothed catfish along the stream of Mariotteya drainage, Egypt [9]. In the present case, *C. parapsilosis* and *C. tropicalis* were successfully amplified by sn-PCR. *C. tropicalis* was isolated from expired breathes of captive dolphins and their environments in an aquarium [22] and from healthy shrimp and aquatic mammals found dead in the environment [6]. *C. parapsilosis* is widely distributed as commensal microorganisms of several healthy animal species and in infections reported in different animal hosts (reviewed by Cordeiro *et al.* [7]), but it has not been reported in aquatic organisms. The opportunistic *Candida* spp. invades and colonizes the human epidermis or mucosa after physical damage to epidermal or mucosal layers with consequent multiple ulcerations and gain access for systemic infection [17]. Moreover, *C. parapsilosis* and *C. tropicalis* infections in a communal environment may represent a health hazard especially to immunodeficient individuals, children and elderly humans. In medical field, *C. tropicalis* is even more invasive than *C. albicans* particularly in immunocompromised patients [14, 19, 24]. Although the source of infection of *C. parapsilosis* and *C. tropicalis* in fish has not been determined in the present study, probably organic materials in aquatic environment might be source of infection. Yeasts have incidence in diverse ecosystems such as soils, plants, animals and other organic matter in aquatic and marine environments [13]. Because of zoonotic potentials of *Candida* spp. *C. albicans* and *C. tropicalis*, workers and dealers with fresh samples of infected fishes should follow up the hygienic measures while collecting infected samples. *Candida* spp. *C. albicans* and *C. tropicalis* have been isolated from some dolphins, staff and aquarium environment [22].

Histologically, multiple disseminating granulomatous lesions in gills, liver and kidneys might indicate the invasiveness of such types of mycoses. Gills were suspected as the primary site of the infection, as they in contact with the outer environment. The lesions seemed to spread hematogenously to the liver and kidneys. Ischemic lesions related to thrombosis are assigned to be the primary site of infection in the present case. Lesions of necrosis in secondary lamellae might be a part of an ischemic lesion that not seen enough in the histological section or might be the initial lesion of the yeast infection that progress to the massive ischemic lesion. Disseminated candidiasis due to *C. albicans* was observed in five captive cetacean including; two adult males of Atlantic bottlenose dolphin (*Tursiops truncatus*) and belukha whale (*Delphinapterus leucas*), a juvenile female harbor porpoise (*Phocoena phocoena*) and a juvenile male longfinned pilot whale (*Globicephala melaena*) [8]. Similar granulomatous lesions were observed in liver, heart and spleen as well as small intestine serosa in Amazon parakeets (*Amazona aestiva*) due to disseminated *C. albicans* infection [3].

Molecular detection of *Candida* spp. infection using sn-PCR has been previously used in human [2] and has been used to differentiate between different species of *Candida* [4]. Sn-PCR is a specific and sensitive method for the diagnosis of human candidemia caused by *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata* [12]. In addition, sn-PCR is a rapid technique and has an advantage of identifying samples co-infected with more than one *Candida* species [16].

The diagnosis of *Candida* spp. infections depends on the demonstration of budding yeast cells and pseudohyphae (or true hyphae) in tissues and on the isolation of the microorganism. In the present case, the causative agent was not definitive by histopathology and immunohistochemistry, but *Candida* spp. infection was the most suspect. Further molecular investigation of sn-PCR clarified *C. parapsilosis* and *C. tropicalis* as the causative agent. Therefore the molecular investigation was able to identify yeast in tissues from the infected fish to the species level. Although the reaction of immunostaining using anti-*C. albicans* antibody was mild and faint staining, the molecular detection of *C. albicans* was negative, suggesting cross-reactivity between *C. albicans* antibody and other *Candida* spp. antigens. Molecular detection using sn-PCR technique on FFPE tissue to identify *C. parapsilosis* and *C. tropicalis* in fish tissue might be an appropriate method to diagnose mycotic infections in archival tissues and when fresh mycological culture is not available. To the author's knowledge, this case is first report of disseminated mycotic granulomatous lesion due to *C. parapsilosis* and *C. tropicalis* infection in fish species.

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