Medical Mycology Case Reports 9 (2015) 7–11

Contents lists available at ScienceDirect

Medical Mycology Case Reports

journal homepage: www.elsevier.com/locate/mmcr

Skin and subcutaneous mycoses in tilapia (*Oreochromis niloticus*) caused by *Fusarium oxysporum in coinfection with Aeromonas hydrophila*

M. Teresa Cutuli [a,1], Alicia Gibello [a,*,1], Antonio Rodriguez-Bertos [b], M. Mar Blanco [a], Morris Villarroel [c], Alejandra Giraldo [d], Josep Guarro [d]

[a] Department of Animal Health, Faculty of Veterinary Sciences, Complutense University, Madrid 28040, Spain
[b] Department of Internal Medicine and Animal Surgery, Faculty of Veterinary Sciences, Complutense University, Madrid 28040, Spain
[c] Department of Animal Science, E.T.S.I.A. Polytechnic University of Madrid, Madrid 28040, Spain
[d] Mycology Unit, School of Medicine and Health Sciences, Institut d'Investigació Sanitària Pere Virgili, Universitat Rovira i Virgili, Reus 43201, Spain

ARTICLE INFO

Article history:
Received 15 April 2015
Received in revised form
4 June 2015
Accepted 5 June 2015
Available online 12 June 2015

Keywords:
Fusarium oxysporum
Nile tilapia
Skin mycoses
Subcutaneous mycoses

ABSTRACT

Subcutaneous mycoses in freshwater fish are rare infections usually caused by oomycetes of the genus *Saprolegnia* and some filamentous fungi. To date, *Fusarium* infections in farmed fish have only been described in marine fish. Here, we report the presence of *Fusarium oxysporum* in subcutaneous lesions of Nile tilapia (*Oreochromis niloticus*). Histopathologic evaluation revealed granuloma formation with fungal structures, and the identity of the etiological agent was demonstrated by morphological and molecular analyses. Some of the animals died as a result of systemic coinfection with *Aeromonas hydrophila*

© 2015 International Society for Human and Animal Mycology. International Society for Human and Animal Mycology Published by Elsevier B.V. All rights reserved.

1. Introduction

The genus *Fusarium* encompasses a wide diversity of species, usually found in air, soil, plants, sea water and freshwater [1–3]. But in recent years, *Fusarium* infections are becoming an important human health problem in immunocompromised patients [4]. In animals, *Fusarium* infections have also been reported in several aquatic animals such as sharks, dolphins, whales, shrimp, in addition to amphibians and reptiles [5]. With the development of aquaculture, fish fungal infections have also increased, due in large part to the stressful conditions of fish farming [6,7]. In fact, fungal infections in fish are generally considered secondary to some other environmental factor (water quality problems, changes in salinity or temperature, trauma) or pathogen (bacterial disease or parasites).

The genus *Saprolegnia* and other typical water molds are often involved in infections of wild and farmed freshwater fish, and some fungi such as *Aphanomyces* and *Fusarium* are also considered important fish pathogens affecting marine fish and shellfish [8–10]. Fusarial infections in marine fish include deep mycoses, ocular and skin lesions, fatal ulceration and necrohemorrhagic dermatitis [6].

Nile tilapia (*Oreochromis niloticus*) is one of the most commonly cultured fish species worldwide because of their high protein content, rapid growth and good palatability. Skin infections in tilapia are mainly caused by bacteria, parasites and *Saprolegnia* species [7,11], but these fish are also susceptible to other species of oomycetes and soil fungi [7]. Here, we report the first case of tilapia infection by *Fusarium oxysporum* species complex confirmed by culture, molecular identification and histopathology.

2. Case

A group of 2000 tilapia fry were bought from a local fish farm weighing 2.16 g ± 0.36 (4 cm ± 0.5 long) and were held in 400 L stock tanks in a recirculating system at the Animal Production Field Station of the Universidad Politécnica de Madrid (UPM) (day 0). The water temperature was 24.6 °C, pH was 7.02 and the mean ammonia levels were 0.10 ppm. The facilities were in good condition and the quality of the water was checked periodically and showed no drastic changes in temperature or other parameters that could affect the fish. One week later (day +7), 20 fish were

* Corresponding author.
E-mail address: gibelloa@vet.ucm.es (A. Gibello).
[1] These authors contributed equally to this work

http://dx.doi.org/10.1016/j.mmcr.2015.06.002
2211-7539/© 2015 International Society for Human and Animal Mycology. International Society for Human and Animal Mycology Published by Elsevier B.V. All rights reserved.

transferred to one of four 100 L tanks for a feeding trial with different amounts of propolis sprayed onto commercial pelleted feed (Skretting feed D2 Alterna Basic, 32% protein). Fish were fed at 10% live weight, twice per day. Eight weeks after their arrival (day +56), some fish began to present abnormal reddening above the eyes and hemorrhagic subcutaneous spot lesions on the head or on the top part of the opercula as if the scales had been removed. Two weeks later (day +70), all fish used in the trial and 50% of those maintained in the stock tanks had died.

Examination of moribund and dead fish revealed the presence of fungal lesions on the skin of the head (Fig. 1 A). Based on this observation, surviving fish were treated with methylene blue (0.5 ppm/L) and malaquite green (0.2 ppm/L) dissolved in the water for four days.

Ten moribund fish were euthanized with tricaine methanesulfonate (MS222, 250 mg/L), and several tissue samples were obtained aseptically at necropsy for microbiological study. All fish procedures were performed according to European Union legislation in relation to animal care. Necropsied fish displayed multiple petechial hemorrhages in the serosa of the celomic cavity and the presence of a green ascitic fluid in the abdominal cavity (data not shown). For histopathological studies, after necropsy, fish were fixed by immersion in 10% buffered formalin, stabilized with methanol at pH 7 for 24 h at room temperature, embedded in paraffin, sliced into 4 μm sections, routinely stained with haematoxylin and eosin, and examined under light microscopy. For microbiological analyses, samples from the skin lesions were streaked onto Sabouraud dextrose agar (SDA) and Columbia 5% sheep blood agar plates (CBA), and cultures were incubated at 25 ± 1 °C for seven days. Also, swabs of the internal organs (kidney and liver) were cultured on CBA and incubated at 29 ± 1 °C for 72 h.

Histopathological evaluation revealed a marked congestion of the hepatopancreas, with small foci of necrosis of hepatocytes associated with neutrophilic reaction (multifocal necrotizing hepatitis), and the presence of bacteria-like elements inside the macrophage cells (Kupffer cells and activated circulating monocytes). In some animals, massive liver necrosis with absence of inflammatory reaction was observed (data not shown). The spleen parenchyma showed increased cellularity due to infiltration of numerous neutrophils and decreased number of

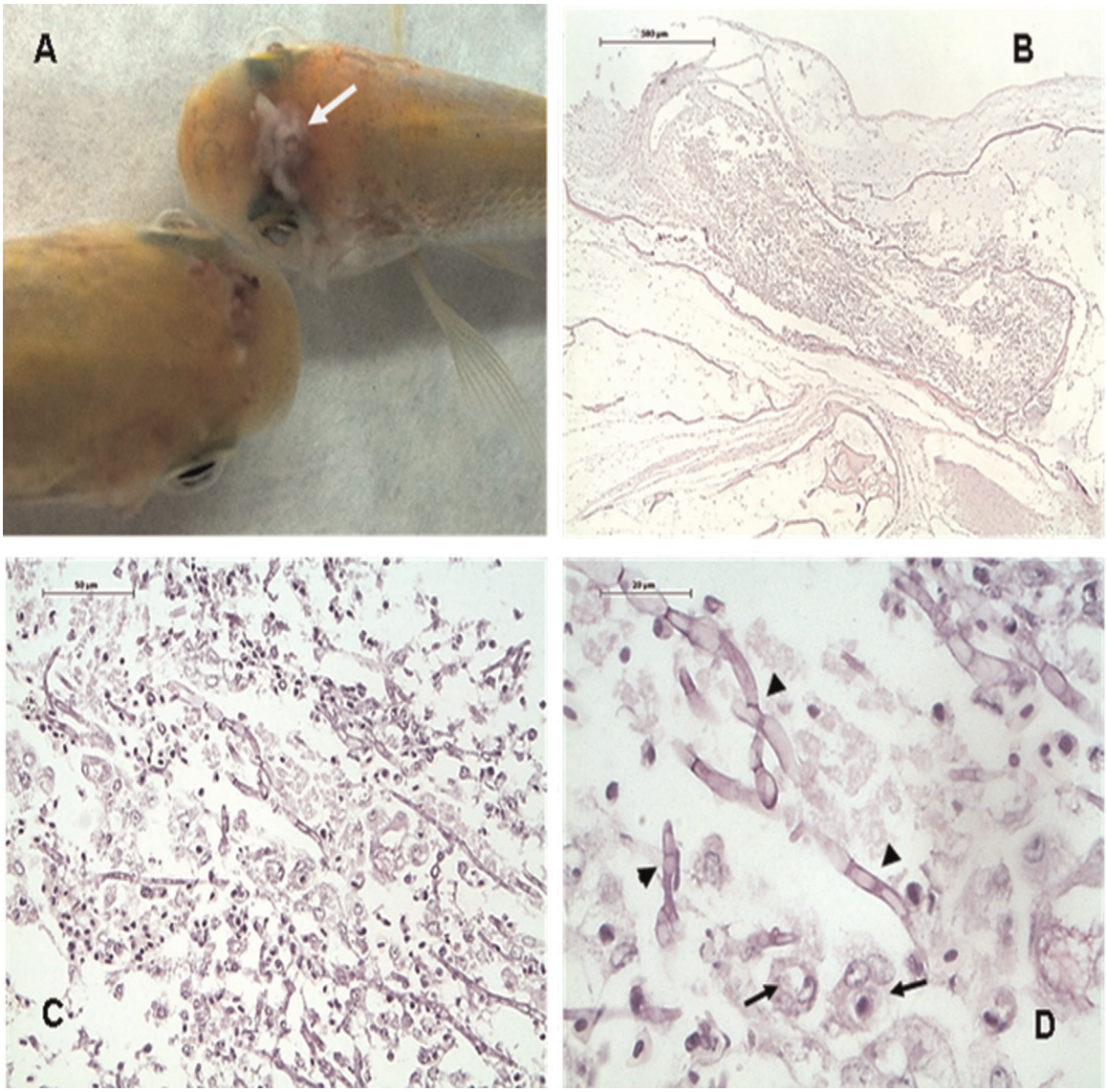

Fig. 1. (A) Gross appearance of the head and skin lesions of fish: soft creamy and yellowish nodules with hyphae and hemorrhagic subcutaneous spot; (B) histological appearance of nodules with low magnification. H&E. 2,5 × –Bar – 500 μm; (C) skin granuloma formation composed of numerous foamy macrophages, numerous neutrophils and fungal formations compatible with septate hyphae and conidia. PAS. 10 × – Bar – 50 μm. (D) Dermal fungal structures with high magnification: septate hyphae (head arrows) and intracytoplasmatic conidia (arrows) into the macrophages. PAS. 40 × – Bar – 20 μm.

melanomacrophage centers. Renal acute tubular necrosis was observed in proximal convoluted tubules. In the renal interstitium, hemorrhagic foci could be observed, as well as an increase in the number of melanomacrophage aggregates. Some of these macrophage cells also exhibited phagocytic vacuoles in the cytoplasm. There was a moderate edema of the corium of the secondary gill lamellae, with marked cell desquamation and mild to moderate infiltration of neutrophils and round cells. In the telencephalon, the main histopathological lesions were submeningeal hemorrhage, neuropil spongiosis and edema in the space of Virchow. At skin level, there was an obvious spongiosis lining epithelium, appreciating exophytic formations in the region of the head corresponding to inflammatory granulomes composed of abundant neutrophils, macrophages and compatible fungal hyphae and conidia (associated with pyogranulomatous reaction) (Fig. 1B–D).

Fungal cultures isolated from the skin lesions on SDA yielded colonies morphologically compatible with *Fusarium* species. Plates were submitted to the Mycology Unit, Medical School and IISPV of the Universitat Rovira i Virgili (URV, Reus) for further identification and characterization. There, fungal colonies were transferred to potato dextrose agar (PDA), oatmeal agar (OA) and synthetic nutrient agar (SNA), and incubated for three weeks at 25 °C. The color of the growing colonies was rated according to the color chart of Kornerup and Wanscher [12], and microscopic features were observed by making direct wet mounts with 85% lactic acid and Shear's solution. The fungal isolate produced cottony and fast growing colonies on PDA (Fig. 2A), white with deep violet (15E8) shades (obverse and reverse); conidiogenous cells were monophialidic, 10–20 × 2–3 μm, (Fig. 2 B) microconidia were hyaline, one-celled, ellipsoidal, cylindrical or reniform, 4–7 × 2–3 μm, arranged in false heads (Fig. 2 C and D); macroconidia were hyaline, usually 3-septate, slightly falcate to almost straight, 11–22 × 3–4 μm; and chlamydospores were abundant on OA and SNA, hyaline, globose or subglobose, 5–9 × 5–8 μm, terminal or intercalary, singly or in pairs (Fig. 2 E). These macroscopic and microscopic features were compatible with a *Fusarium* species.

For the molecular identification, genomic DNA was extracted from fungal colonies growing on PDA. A partial fragment of the translation elongation factor 1 alpha gene (*TEF-1α*) was amplified with the primer set EF-1H/EF-2T, following the protocol described by O'Donnell et al. [13]. The PCR product was purified and sequenced at Macrogen Corp. Europe (Amsterdam, the Netherlands).

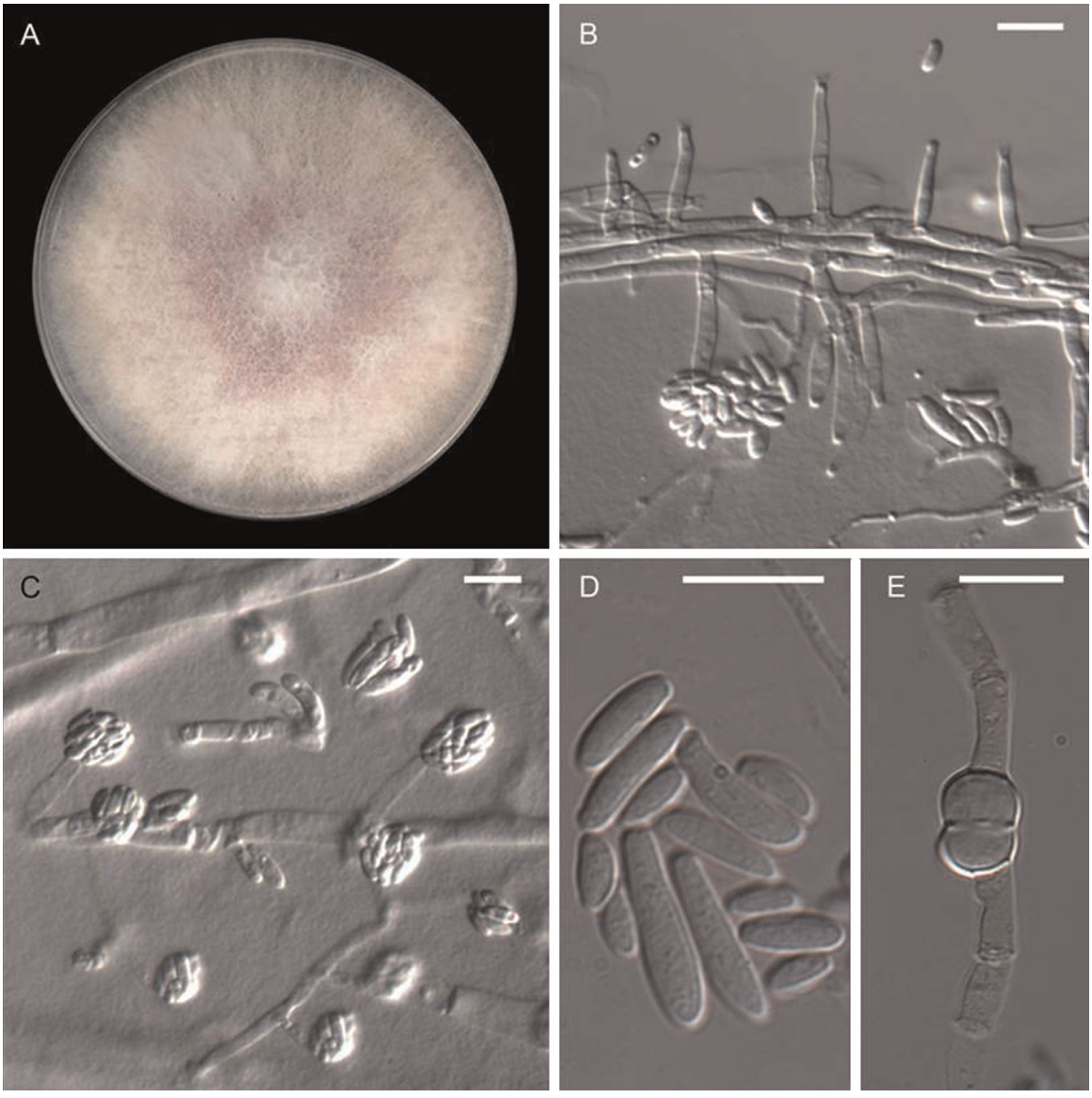

Fig. 2. Macroscopic and microscopic features of *Fusarium oxysporum* species complex isolate FMR 13411. (A) Colony on PDA after 14 days at 25 °C. (B) Monophialides. (C) Microconidia arranged in false heads. (D) Mesoconidia and microconidia (E) Intercalary chlamydospores. Scale bar = 10 μm.

The sequencing was carried out with the same primers used for amplification to ensure good quality sequences over the total length of the amplicon. Consensus sequences were obtained using SeqMan version 7.0.0 (DNASTAR, Madison, WI, USA). The BLAST search result at the *Fusarium*-ID database (isolate.fusariumdb.org) [14] showed that the *TEF-1α* sequence from the clinical isolate was 100% identical with several isolates of the *Fusarium oxysporum* species complex 33, i.e. NRRL 38515 (FD_00711_EF-1a), NRRL 38360 (FD_00635_EF-1a) and NRRL 36153 (FD_00431_EF-1a). The isolate was stored in the culture collection of the Medical School (URV, Reus) under the code FMR 13411. The nucleotide sequence corresponding to the *TEF-1α* encoding gene has been assigned the accession number KP893745 in the GenBank/EMBL database.

Gram negative, oxidase positive coccobacilli were recovered in pure culture from the the internal organs on CBA of all analyzed fish. The identification of the isolates was performed by using the API 20NE system (bioMérieux) and by PCR, using the universal primers for the *16S rRNA* gene: AGAGAGTTTGATCATGGCTCAGGA (forward) and the primer GGTTACCTTGTTACGACTT described previously [15], as reverse. The clinical isolate was identified as *Aeromonas hydrophila/caviae.* The PCR-fragment (approximately 1400 bases) was referred to Secugen (Sequencing Facilities, Madrid), and its analysis revealed 99% sequence identity with the type strain of *A. hydrophila* ATCC7966 (accession number NR119039).

3. Discussion

Fusarium is a large genus that belongs to the Ascomycota phylum and comprises a few hundred species that are mainly distributed in soils and in water systems [1,3]. *Fusarium solani*, *F. oxysporum* and *F. verticillioides* have been reported as the most frequent species causing invasive fusariosis in humans, especially in immunocompromised patients [4,16,17]. Immunocompetent individuals can be also affected, but in these patients, *Fusarium* usually causes localized infections. Fungal infections in fish are generally considered secondary to other pathogens (bacterial infections or parasites) or related with water or environmental changes that can cause stress to the animals. Fungi, however, can cause disease under a variety of other circumstances, and some of them may be more aggressive and play a more primary role in infectious diseases [5,6]. *Fusarium* infection in fish usually causes dermatitis and systemic lesions and the progression can vary from several days to weeks, depending upon other factors (e.g. water quality or natural sunlight) [6]. In the present case, mortalities began eight weeks following arrival, hence immunosuppression secondary to transport did not seem to predispose the epizootic. Also, the disease was not related to the feeding trial since both fish used in the trial and those maintained in the stock tanks were affected.

Fusarial infections in marine fish include localized epidermal lesions with skin defects and ulcers [5,6]. Infections can be aggressive, often leading to invasion of underlying muscle and bone, and in several cases, becoming systemic and infecting the kidney and brain. Severe chronic granulomatous dermatitis, cellulitis, and myositis with fungal elements can be seen on histopathology [6]. However, organ system involvement in *Fusarium* infections is absent or limited to only a small number of cases [5]. In the only infection by *F. oxysporum* in farmed fish reported previously, no external signs were observed, but kidneys were remarkably swollen and discolored [8]. The tilapia analyzed in the present case showed lesions both externally and internally, in the region of the head. Moreover, since some animals showed hemorrhagic subcutaneous spot lesions, without hyphal growth on the surface of the skin, it appears that the progression of fungal growth was from inside to outside.

The combination of skin lesions and positive bacterial cultures involving other sites is the most frequent clinical presentations of disseminated fusariosis in humans and animals [6,18]. Although *A. hydrophila* is typically recognized as an opportunistic pathogen, it also causes severe outbreaks of disease of pond-cultured and wild fresh-water fishes [19]. In the case described here, a septicemic infection by *A. hydrophila* was confirmed by culture, DNA sequencing and histopathological findings in the liver and kidney of fish (data not shown), which could have been favored by the fungal infection. A mixed *A. hydrophila* and *Fusarium* spp. infection in ornamental fish *(Symphysodon* spp.) has been reported recently [20]. In both cases, it appears possible that *F.oxysporum* might have acted as the primary invader, promoting bacterial infection by tissue damage. In fact, treatment of surviving tilapia with methylene blue and malaquite green decreased the mortality rate, probably acting on the fungus directly and preventing the infection by oportunistic bacteria.

Fusariosis has been reported sporadically in fish and other aquatic organisms, but identification has often been limited to the genus level, or based solely on morphologic features [5,20]. The identification of *Fusarium* at the species level is often difficult and requires a specialized laboratory and skilled personnel. The distribution of *F. oxysporum* is world-wide and it occurs chiefly as a soil saprophyte. Therefore, it is possible that this species could have been involved in more superficial and deeper infections in human and animals than those reported. Accurate identification of fungal pathogens, including those that cause infections in both animals and humans, such as *Fusarium*, is valuable in terms of epidemiological studies of those processes and in terms of clinical management [5,18]. To our knowledge, this report is the third description of *F. oxysporum* infection in fish, and the first one in a farmed freshwater fish.

Conflict of interest

There are none.

Acknowledgments

This study was supported by the Projects AGL2012-35419 from the Spanish Ministry of Economy and Competitiveness and S2013/ABI-2747 from Community of Madrid (CAM). Authors thank JP. de la Roche and F. Berdejo for their help with the animal care.

References

[1] Nelson PE, Dignani MC, Anaissie EJ. Taxonomy biology, and clinical aspects of *Fusarium* species. Clin. Microbiol. Rev. 1994;7:479–504.
[2] Michielse CB, Rep M. Pathogen profile update: *Fusarium oxysporum*. Mol. Plant Pathol. 2009;10:311–24.
[3] Palmero D, Iglesias C, Cara M, de, Lomas T, Santos M, Tello JC. Species of *Fusarium* isolated from river and seawater of southeastern Spain and pathogenicity on four plant species. Plant Dis. 2009;93:377–85.
[4] Peterson A, Martin H, Lee PH, Commins D, Cadden J, Giannotta SL, et al. Intracranial *Fusarium* fungal abscess in an immunocompetent patient: case report and review of the literature. J. Neurol. Surg. Rep. 2014;75:e241–5.
[5] Salter CE, O'Donnell K, Sutton DA, Marancik DP, Knowles S, Clauss TM, et al. Dermatitis and systemic mycosis in lined seahorses *Hippocampus erectus* associated with a marine-adapted *Fusarium solani* species complex pathogen. Dis Aquat Organ 2012;101:23–31.
[6] Yanong RP. Fungal diseases of fish. Vet. Clin. Exot. Anim. 2003;6:377–400.
[7] Ali EH, Hashem M, Al-Salahy MB. Pathogenicity and oxidative stress in Nile tilapia caused by *Aphanomyces laevis* and *Phoma herbarum* isolated from farmed fish. Dis. Aquat. Organ. 2011;94:17–28.
[8] Hatai K, Kubota SS, Kida N, Udagawa S. *Fusarium oxysporum* in red sea bream (*Pagrus* sp.). J. Wildl. Dis. 1986;22:570–1.

[9] Hatai K. Diseases of fish and shellfish caused by marine fungi. In: Raghukumar C, editor. Biology of Marine Fungi, Progress in Molecular Subcellular Biology, 54. Berlin Heidelberg: Springer-Verlag; 2012. p. 15–52.
[10] Oidtmann B. Review of biological factors relevant to import risk assessments for epizootic ulcerative syndrome (*Aphanomyces invadans*). Transbound. Emerg. Dis. 2012;59:26–39.
[11] El-Sharouny HM, Badram RA. Experimental transmission and pathogenicity of some zoosporic fungi to Tilapia fish. Mycopathologia 1995;132:95–103.
[12] Kornerup A, Wanscher JH. Methuen Handbook of Colour. 3rd ed.. London: Eyre Methuen; 1978.
[13] O'Donnell K, Cigelnik E, Nirenberg HI. Molecular systematics and phylogeography of the *Gibberella fujikoroi* species complex. Mycologia 1998;90:465–93.
[14] Geiser DM, Jimenez-Gasco M, Kang S, Makalowska I, Veeraraghavan N, Ward TJ, et al. FUSARIUM-ID v. 1.0: A DNA sequence database for identifying *Fusarium*. Eur. J. Plant Pathol. 2004;110:473–9.
[15] Borrell N, Acinas SG, Figueras MJ, Martínez-Murcia AJ. Identification of *Aeromonas* clinical isolates by restriction fragment length polymorphism of PCR-amplified 16S rRNA genes. J. Clin. Microbiol. 1997;35:1671–4.
[16] Guarro J. Fusariosis, a complex infection caused by a high diversity of fungal species refractory to treatment. Eur. J. Clin. Microbiol. Infect. Dis. 2013;32:1491–500.
[17] Latif A-L, Ch Harper, Mcdonald I, Morrison A. Splenectomy as an effective debulking therapy for disseminated mould infection in acute myeloid leukaemic following adjuvant therapy with interferon gamma and liposomal amphotericin. Med. Mycol. Case Rep. 2012;1:82–4.
[18] Souza M, de, Matsuzawa T, Lyra L, Busso-Lopes AF, Gonoi T, Schreiber AZ, et al. *Fusarium napiforme* systemic infection: case report with molecular characterization and antifungal susceptibility tests. Springerplus 2014;3:492.
[19] Roberts RJ. The bacteriology of teleosts. In: Roberts RJ, editor. Fish Pathology. London: Saunders; 2001. p. 315–8.
[20] Abd El-Ghany NA, El-khatib NR, SSA Salama. Causes of mortality in Discus fish (Symphysodon) and trials for treatment. Egypt. J. Aquac. 2014;4:1–12.