Proceedings

The Seventh International Conference on

Recirculating Aquaculture

July 25–27, 2008

The Hotel Roanoke & Conference Center
Roanoke, Virginia

The biennial international conference and trade show

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The Assessment of Mycotic Settlement of Freshwater Fish in Egypt

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Introduction

Many of the fungi that affect fish are considered opportunists, attacking fish when they are stressed or immunocompromised because of unfavorable environmental conditions, or secondary to bacterial or viral infections, or when they have lost their mucus protection because of trauma or excessive handling (Roberts, 1989; Quiniou et al., 1998). Mycotic infections of fish by Oomycetes are wide spread in freshwater and represent the most important fungal group affecting wild and cultured fish. The *Saprolegniaceae*, specifically members of the genus *Saprolegnia*, are responsible for significant infections involving both living and dead fish and eggs. Oomycetes are classical saprophytic opportunists, multiplying on fish that are physically injured, stressed or infected (Pickering and Willoughby,1982). Members of this group are generally considered agents of secondary infection arising from conditions such as bacterial infections, poor husbandry, and infestation by parasite and social interaction. However, there are several reports of Oomycetes as primary infectious agents of fish (Pickering and Christie, 1980) and their eggs (Walser and Phelps, 1993). Moreover, there are other fungi that have been implicated in fish diseases. Some of the genera involved include *Aspergillus* (Salem et al., 1989b), *Fusarium* (Bisht et al., 2000), *Ichthyophonus* (Faisal et al., 1985), *Branchiomyces* (Easa et al., 1984), *Phoma* (Hatai et al., 1986), *Paecilomyces* (Lightner et al., 1988), *Exophialia* (Langdon and MacDonald, 1987), *Phialophora* (Ellis et al., 1983), *Rhizomucor* (Wolf and Smith, 1999) and *Candida* (Neish and Hughes, 1980). Most of these are multiple case reports or single, and causing systemic disease with high mortality rates in fish. The objectives of this study are to determine the types of fungal pathogens affecting freshwater fish especially those causing high mortality rates, elucidate the incidence and distribution of such pathogens in *Oreochromis* species and *Clarias gariepinus*, study the seasonal variations enhancing fungal diseases of fish and determinate the pathogenicity of the most prevalent isolated fungi.

Materials and Methods

Three hundred sixty fish were observed for their behavior and external lesions prior to autopsy. Then they were killed and examined. The examination included external changes as well as examination of internal organs. Wet mount preparation of fish samples were commonly made in 10% KOH. A simple stain such as lactophenol cotton blue was used. The preparation was examined microscopically after about 30 minutes for fungal elements. Mycological examination

was done according to and (Noga, 1993). Identification of moulds was carried out according to Refai (1987). Identification of yeasts: Plates were examined microscopically for the presence of chlamydospores, arthrospores and blastospores (Refai, 1987) and the scheme of identification of yeasts given by Terrence (1971). Urease test was conducted as specified by Cruickshank et al. (1975). Suspected *Candida* species were scratched onto rice or corn meal agar for pseudohyphae and chlamydospores production (Larone, 1987) and a confirmatory identification was carried out by germ tube test (Martin, 1979).

Histopathological examination: Tissue samples were prepared according to Roberts (1989). and stained by periodic acid Schiff's (PAS) and GMS (Sheehan and Hrapchak, 1980).

Experimental infection: A total of 120 *Oreochromis* species with 30-40 g average body weight were used. They were divided into four equal groups (each one contained 30 fish). Each group was subdivided into three sub-groups, each containing 10 fish.

Preparation of spores suspension for experimental infection: Inocula were prepared by spreading 5 ml of sterile phosphate buffer saline over the plates containing 7-to-10-day-old pure cultures of *Aspergillus flavus* and *Fusarium* sp. The spores were harvested by gentle washing of the surface of the colonies with a sterile loop, then transferred aseptically to sterile flasks. Two drops of tween 80 were added to avoid clumping of spores in the case of the *Aspergillus flavus* group. Spores were counted by aid of haemocytometer and suspension was diluted to reach 9X 10^4 spores/ml for both *Aspergillus flavus* and *Fusarium* sp.

Table. Type, average body weight of fish, spores concentration per ml, dose, route of inoculation and temperature.

Fish	Body weight	Number of fish in each subgroup	Inoculated fungi	Dose	Conc.	Route	Temp.	References
Tilapia sp.	30-40 g	10 10	*Aspergillus flavus*	0.2ml 0.2ml	$9x10^4$ $9x10^4$	I.P I.M	18°C	Olufemi *et al.* (1983)
		5 5	Normal saline	0.2ml 0.2ml	_ _	I.P I.M		
Tilapia sp.	30-40 g	10 10	*Aspergillus flavus*	0.2ml 0.2ml	$9x10^4$ $9x10^4$	I.P I.M	26°C	Olufemi *et al.* (1983)
		5 5	Normal saline	0.2ml 0.2ml	_ _	I.P I.M		
Tilapia sp.	30-40 g	10 10	Fusarium	0.2ml 0.2ml	$9x10^4$ $9x10^4$	I.P I.M	22°C	Muhvich *et al.* (1989)
		5 5	Normal saline	0.2ml 0.2ml	_ _	I.P I.M		
Tilapia sp.	30-40 g	10 10	*Candida albicans*	0.2ml 0.2ml	$2x10^3$ $2x10^3$	I.P I.M	22°C	Faisal *et al.* (1986)
		5 5	Normal saline	0.2ml 0.2ml	_ _	I.P I.M		

Preparation of yeast suspension for experimental infection: A loopfull of one day old pure yeast culture of *Candida albicans* was added to test tube containing 5 ml of sterile phosphate buffer saline and mixed gently to reach equal distribution. Spores were counted by using haemocytometer then suspension was adjusted to reach $2x10^3$ Candida spores per ml.

Results and Discussion

Mycological examination revealed the isolation of 2081 fungal isolates from 150 diseased and 210 apparently healthy fish samples, of which 1334 were isolated from *Oreochromis* species and 747 isolates from *Clarias gariepinus*. Identification of fungi into yeasts and moulds revealed that the percentage of moulds was slightly higher in *Oreochromis* species (80.5%) in comparison to that in *Clarias gariepinus* (78.2 %). On the other hand, the rate of yeast isolates per fish was slightly higher in *Clarias gariepinus*. Isolated moulds belonged to the following genera: Saprolegnia, Aspergillus, Fusarium, Mucor, Penicillium, Rhizopus, Scopulariopsis, Paeciliomyces and Curvularia. The same fungal isolates were reported by Abdel Alim (1992) and Khalil (1993).

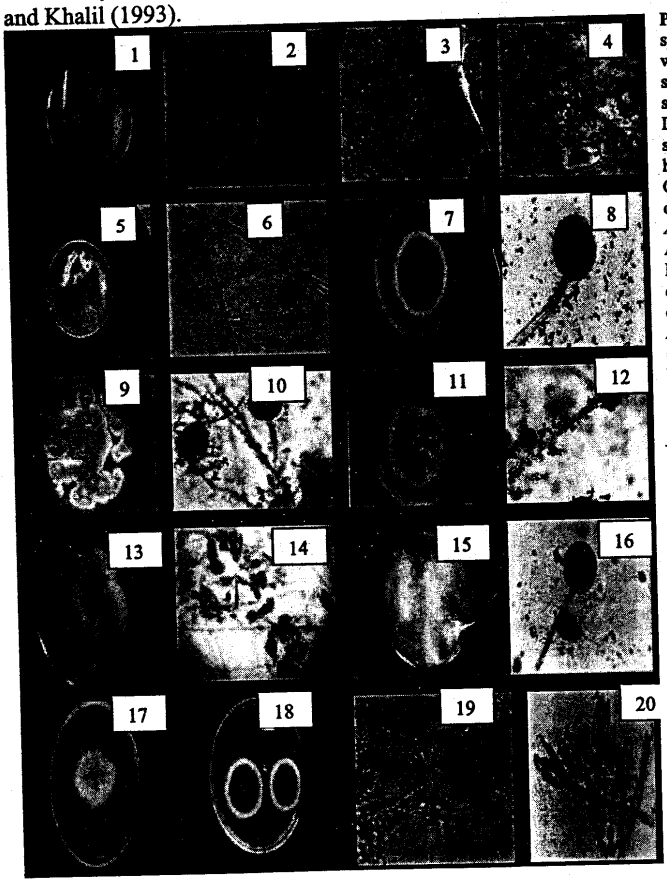


Photo. (1): A colony of *Saprolegnia* species with the characteristic cotton-wool like growth. **Photo. (2):** Non-septated broad hyphae of *Saprolegnia* species (X 200). **Photo. (3&4):** Different stages of reproductive structures of *Saprolegnia* species on hemp seeds (X 400). **Photo. (5):** Colonies of *Aspergillus flavus* on SDA, one week old. **Photo. (6):** Typical heads *Aspergillus flavus* (X 400). **Photo. (7):** A colony of *Aspergillus niger* on SDA. **Photo. (8):** *Aspergillus niger* showing characteristic round head with black conidia (X 400). **Photo. (9):** Colonies of *Aspergillus terreus* on SDA. **Photo. (10):** *Aspergillus terreus* with small hemispherical vesicle (X 400). **Photo. (11):** A colony of *Aspergillus fumigatus* on SDA. **Photo. (12):** *Aspergillus fumigatus* with columnar head (X400). **Photo. (13):** A colony of *Fusarium* species on SDA with rose pigments on the center. **Photo. (14):** *Fusarium* species with characteristic slender, multicelled conidia (X 200). **Photo. (15):** Colonies of *Mucor* species showing spread over the surface of SDA. **Photo. (16):** Round sporangia of *Mucor* species containing sporangiospores (X 400). **Photo. (17):** *Penicillium* species on SDA with different colour and texture. **Photo. (18):** *Penicillium* species showing brush- like arrangement of fruiting head "A" (X400) and "B" (X 200). **Photo. (19):** *Rhizopus* species colony on SDA showing dens woolly mycelia. Sporangia are seen as small black dots. **Photo. (20):** *Rhizopus* species showing long, branched Sporangiophores and terminating with rhizoids (X200).

The Incidence of moulds in diseased and apparently healthy fishes were recorded in (Figures1 and 2), also the incidences of isolated moulds from different organs of *Oreochromis* species (Figure 3) and *Clarias gariepinus* (Figure 4) were detected. Seasonal incidences were seen (Figure 5). These isolates were recovered from apparently healthy and clinically diseased *Oreochromis* species and *Clarias gariepinus* This was expected, as almost all these fungi were categorized by Shaheen (1986) as normal mycoflora. This does not mean that they cannot produce disease. They can better be considered as opportunistic fungi (Refai, 1987) as many of them possess virulence factors, which enable them to cause diseases (Refai et al., 2004), particulary under favourable predisposing conditions. Regarding seasonal incidences, *Saprolegnia* species were isolated with high incidence in winter, followed by early spring and late autumn. This result agrees with Naguib (1994), who stated that the seasonal variations play an important role in spreading of the Saprolegnia infection among freshwater fishes especially during late autumn, winter and early spring, where the water temperature was low.

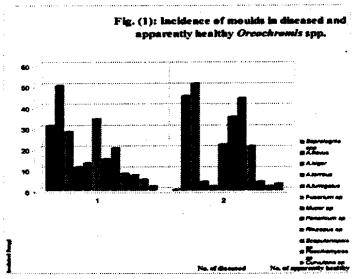


Fig. (1): Incidence of moulds in diseased and apparently healthy *Oreochromis* spp.

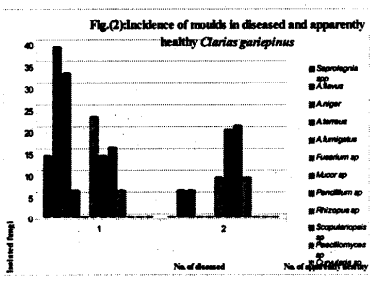


Fig.(2):Incidence of moulds in diseased and apparently healthy *Clarias gariepinus*

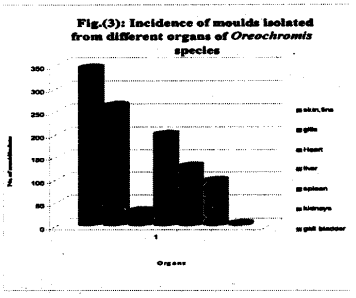


Fig.(3): Incidence of moulds isolated from different organs of *Oreochromis* species

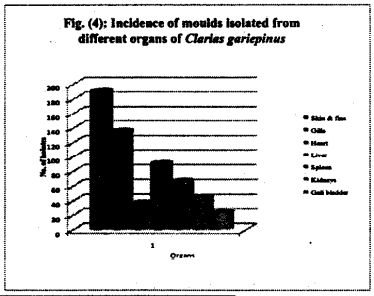
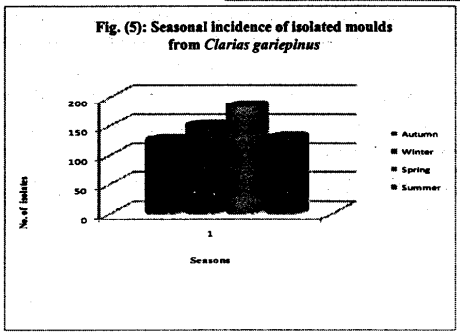


Fig. (4): Incidence of moulds isolated from different organs of *Clarias gariepinus*



Clinical findings of *Oreochromis* species inoculated with *Aspergillus flavus*, *Fusarium* species and *Candida albicans* revealed that exophthalmia (Photo 21), skin darkening (Photo 22), cotton wool-like growth on various parts of the body (Photos 23 and 24), moderate abdominal distention (Photo 25) and corneal opacity and haemorrhages all over the body surface (Photo 26). These resultes are supported by Marzouk et al. (2003).

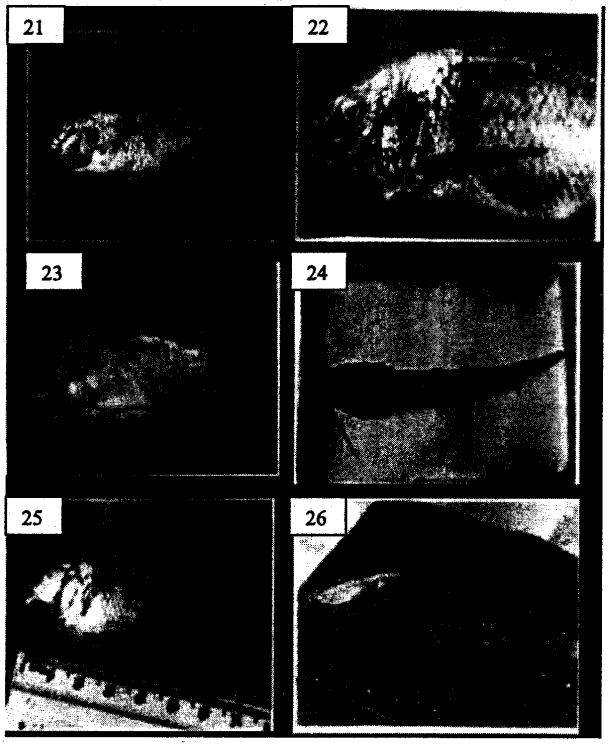


Photo. (21): *Oreochromis* **species showing exophthalmia. Photo. (22):** *Oreochromis* **species showing skin darkening. Photo. (23&24):** *Oreochromis* **species and** *Clarias gariepinus* **showing cotton wool-like growth on various parts of the body. Photo. (25):** *Oreochromis* **species showing ascitis. Photo. (26):** *Clarias gariepinus* **showing haemorrhages allover the body surface.**

Postmortem findings revealed congestion and ulceration of gills (Photo 27), haemorrhagic abdominal fluids, necrotic foci within liver and distention of gall bladder (Photo 28), multiple nodules within spleen (Photo 29) and severe intestinal congestion (Photo 30). On the other hand, no clinical or postmortem changes were detected on fish groups maintained at 18°C. These findings are in agreement with those of Refai et al. (1987).

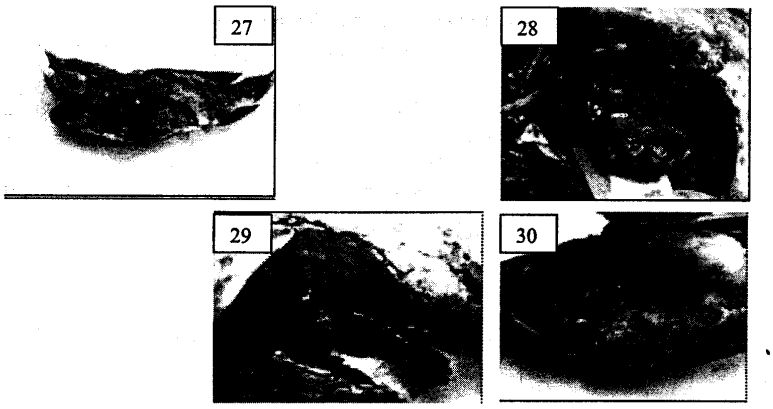


Photo. (27): Liver of *Oreochromis* species showing necrotic foci with distention of gall bladder. **Photo. (28):** Spleen of *Oreochromis* species showing multiple nodules **Photo. (29):** *Oreochromis* species showing severe enteritis. **Photo. (30):** *Oreochromis* species showing severe enlargement of spleen.

The pathological changes and the fungal elements in tissue sections in naturally infected fishes of various organs are described under each of the following photos (31-39), stained by either PAS or GMS stains.

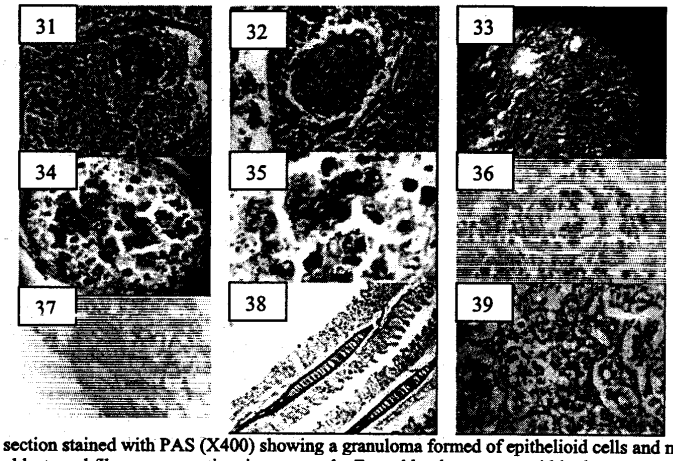


Photo. (31): Spleen section stained with PAS (X400) showing a granuloma formed of epithelioid cells and macrophages surrounded with fibroblasts and fibrous connective tissue capsule. Fungal hyphae appear within the granuloma. **Photo. (32):** Spleen section stained with PAS (X400) showing granuloma consists of epitheloid cells, macrophages and surrounded with connective tissue capsule. Large number of fungal spores appear within and surrounding granuloma.**Photo. (33):** Liver section showing fungal hyphae between the hepatocytes stained with PAS (X200). **Photo. (34):** Liver section stained by GMS (X400) showing granuloma consisting of an aggregation of epithelioid cells, macrophages and fibrous connective tissue capsule. Fungal hyphae and spores appear within granuloma. **Photo. (35):** Liver section stained by GMS (X 1000) showing fungal hyphae and spores between the hepatic tissue. **Photo. (36):** Spleen section stained by GMS (X 400) showing focal aggregation of spores surrounded with proliferating fibroblasts and fibrous connective tissue in between. **Photo. (37):** Kidney section stained by GMS (X 400) showing hyphal threads in between the interstitial tissues with marked severe degenerative changes in the tubular epithelium. **Photo. (38):** Gills section stained by GMS (X 400) showing yeast cells investing necrosed areas of epithelial lining the secondary lamellae. **Photo. (39):** Kidney section stained by GMS (X 400) showing yeast cells investing the interstitial tissues.

It can be concluded from the results obtained in the present work that, though most fungi isolated from fish are considered by several authors as normal mycoflora, we found in the present study that many fungi can cause natural infections. This was confirmed by histopathological reactions characteristic of fungal infection in naturaly infected fish, and the presence of fungal elements in the lesions. This was substantiated also by experimental infection of fish, that induced similar findings as the natural infection, i.e. a clear application of Koch's postulate. This conclusion should direct our attention to the possible role of fungi in affecting the fish industry.

Referances

Abdel-alim, K. 1992. The role of fish in transmitting some bacterial and fungal diseases to man. M.V.Sc. Thesis, Faculty of Vet. Med., Alexandria University.

Bisht, D., G.S. Bisht, and R.D. Khulbe. 2000. Fusarium A new threat to fish population in reservoirs of Kumaun India .Curr. Sci. 78(10):1241-1245.

Cruickshank, R., J.P. Dauguid, B.P. Marmion, and R.H.A. Swain. 1975. "Medical Microbiology" Twelfth Edition. Volume ÉÉ. Pub. Churchill Livingston. Eding Urgh, London.

Easa, M. EI-S, M.E. Hatem, E.E. Sark, and M. Refai. 1984. *Phoma herbarum* as a mycotic fish pathogen in *Clarias lazera*, Armout catfish. Vet. Med. J. 32:257-267.

Ellis, A.E., I.F. Waddell, and D.W. Minter. 1983. A systematic fungal disease in Atlantic *salmon parr*, *Salmo salar L.*, caused by a species of Phialophora. J. Fish. Dis. 6:511-523.

Faisal,M., H. Torky, and H.H. Reichenbach-Klinke. 1985. A note on swimming disease among the Labyrinth catfish (*Clarias lazera*). J. Egypt. Assiut Vet. Med. 45:53-60.

Faisal, M., M.E. Easa, H. Torky, and F. El-Sltatoury. 1986. Cryptococcosis in cultured Nile perch (*Lotus niloticus*) *Alex.* J. Vet. Sci. 2(1):405-417.

Hatai, K., Y. Fujimaki, and S. Egusa. 1986. A visceral mycosis in ayu fry, Pleoglossus altivelis Temminck & Schlegel, caused by a species of Phoma. J. Fish. Dis. 9:111-116.

Khalil, R.H. 1993. Some studies on mycotic infection in some freshwater fish with special reference to its control. Thesis ; M.Sc.; Fish Diseases & Hygiene Alex. Univ. Fac. of Vet. Med.

Langdon, J.S. and W.L. MacDonald. 1987. Cranial *Exophilia piscphilia* infection in *Salmo salar* in Australia. Bull. Eur. Ass. Fish Pathol. 7:35-37.

Lightner, D., R.M. Redman, L. Mohney, J. Sinski, and D. Priest. 1988. A renal mycosis of an adult hybrid red tilapia, *Oreochromis mossambicus* and *O. hornorum*, caused by the imperfect fungus, *Paecilomyces marquandii*. J. Fish. Dis. 1.1:437-440.

Martin, M.V. 1979. Germ tube formation by oral strains of *Candida albicans*. J.Med. Microbiol. 12:187-193.

Marzouk, M.S., S.R. Samira, and M.H. El-Gamal. 2003. Mycological investigations on cultured Tilapia in Kafer El- Sheikh Governorate. Kafer El-Sheikh Vet. Med. J. 1(2):97-114.

Muhvich, A.G., R. Reimschuessel, M.M. Lipsky, and R.O. Bennett. 1989. *Fusarium Solani* isolated from newborn bone-thead Sharks, Sphyrnatiburo (L). J. Fish Dis. 12:57-62.

Naguib, M. 1994. Studies on bacterial and mycotic affections of freshwater fishes in aquaculture. Ph. D. Thesis, Faculty of Vet. Med., Cairo Univ.

Neish, G.A. and G.C. Hughes. 1980. Diseases of fishes, Book 6. Fungal diseases of fish. TFH Publications, Neptune, Ng.1159.

Noga, E.J. 1993. Fungal and algal disease of temperate freshwater and estuarine fishes, in Stoskopf MK (ed). Fish Medicine. Philadelphia, PA, Saunders, 278-283.

Olufemi, B.X., C. Agius, and R.J. Roberts. 1983. Aspergillomycosis in intensively cultured tilapia. (*Sarothernden* sp.) from Kenya.Vet. Rec. 122:203-204.

Pickering, A.D. and P. Christie. 1980. Sexual differences in the incidence and severity of ectoparasitic infestation of the brown trout, *Salmo trutta L.* J. Fish Biol. 16:669–683.

Pickering, A.D. and L.G. Willoughby. 1982. Saprolegnia infections of salmonid fish. In Microbial Diseases of Fish, Edit Roberts, R.J. Academic Press, London, pp. 271–298.

Quiniou, S.M.A., S. Bigler, and L.W. Clem. 1998. Effects of water temperature on mucous cell distribution in channel catfish epidermis: a factor in winter saprolegniasis. Fish Shellf Immunol. 8:1-11.

Refai, M. 1987. Isolation and identification of fungi. Fac. Vet. Mid. Cairo University.

Refai, M., M.M. Abdel Halim, M.M.H Afify, H. Youssef, and K.M. Marzou. 1987. Studies on aspergillomycosis in catfish (*Clarias Lasera*). Allgemeine Pathologic and pathologische Anatomic. Tagung der Deutachen Veterinar - Medizinischen Gesellschaft. der Europeischen Gesellschaft fur Vet. Pathol. 63:1-12.

Refai, M., S. Attia, R.M. Salem, and E.M. El-Dahshan. 2004. Studies on the pathogenicity of *Aspergillus fumigatus*, *A. flavus* and *A. niger* isolated from chickens and their enviroment. Egypt. J. Comp. Path., Clinic. Path. 17(2):193-205.

Roberts , R.J. 1989. The mycology of teleosts, in Roberts RJ (ed). Fish Pathology, 2nd edition. London, England, Baillere Tindall, pp 320-336

Salem, A.A.. M.K. Refai, I.A.M. Eissa, M. Marzouk, A. Bakir, M. Moustafa, and Manal Adel. 1989b. Some studies on aspergillomycosis in Tilapia nilotica Zagazig Vet. J., 17(3):315-328.

Shaheen, A.A. 1986. Mycoflora of some freshwater fish. M.V.Sc. Thesis, Fac. Vet. Med., Zagazic Univ.

Sheehan, D. and B. Hrapchak. 1980. Theory and practice of Histotechnology. 2nd Ed, 164-166, Battelle Press, Ohio.

Terrence, C.D. 1971. A practical approach to identification of yeast like organisms. Amer. Jour. Microbiol., 35(5):580-590.

Walser, C.A. and R.P. Phelps. 1993. The use of formalin and iodine to control Saprolegnia infections on channel catfish, *Ictalurus punctatus*, eggs. J. Appl. Aquacult. 3:269–278.

Wolf, J.C. and S.A. Smith 1999. Systemic zygomycosis in farmed tilapia fish. J. Comp. Pathol. 121(3):301-306.