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Isolation of Mycotoxin-producing Fungi from Fishes Growing in Aquacultures

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ABSTRACT

Contamination of fishes with mycotoxin-producing fungi has a negative impact on the public health. The main goal of this study was to emulate contamination of fishes grown in aquacultures with potentially mycotoxin-producing microfungi. Five fishes including Nile tilapia, African catfish, Tilapia zilli, Bony bream and Thinlip Mullet species were collected from different aquacultures distributed in Delta region, Egypt. From each fish species, at least 10 random samples were subjected for the fungal analysis. The most common isolated fungi were tested for their potentiality to produce mycotoxins *in vitro*. Detection of the mycotoxins was carried out by thin layer chromatography compared to mycotoxin standards. Results showed that 21 fungal species were isolated from five fish species. The highest number of species (15) was isolated from African catfish, but the lowest number (6 species) was isolated from Bony bream. The most common fungal species isolated from these fish species were; *Paecilomyces lilacinus*, *P. variotii* and *Phoma herbarum*. Mycotoxin producing fungi including *Aspergillus flavus*, *A. clavatus*, *A. ochraceus*, *A. parasiticus*, *A. sydowii*, *A. terreus*, *A. versicolor* *Penicillium chrysogenum* and *Trichoderma viride* were recovered. Production of aflatoxin B1, B2 and G1, strigmatocystin ochratoxin and T2-toxin by these fungal species was approved. Most species were found to produce aflatoxin and strigmatocystin while ochratoxin was produced only by *A. sydowii* and *A. versicolor*. This study proved the infection of aquacultures' fishes with mycotoxin-producing fungi. It recommends periodical examination of fishes grown in these aquacultures to ensure that they are devoid of fungal contamination to conserve the public health.

Key words: Aquacultures, fish, fungi, aflatoxin, ochratoxin, strigmatocystin

INTRODUCTION

Fish serve as an important source of human dietary protein worldwide, especially in African countries (Kumolu-Johnson and Ndimele, 2011). Natural fisheries are increasingly exhausted and the aquaculture become important means to compensate the shortage in this important source (Fletcher *et al.*, 1999). At least about 30% of the fish production is coming from aquacultures (Mousavi *et al.*, 2009). Intensive aquaculture conditions can promote the transmission of fish diseases, especially fungal diseases, causing economic losses. Fish diseases are the major cause of limited fish production in aquacultures (Faruk *et al.*, 2004). Moreover, contamination with different mycotoxin producing fungal species is transmitted in aquacultures (Ali *et al.*, 2011). The traditional fungi which produce mycotoxins and/or cause diseases of fish are composed of members from several different taxonomic kingdoms, among which terrestrial fungi represent major taxa.

Terrestrial fungi may enter water distributions from various locations and they can become established in many ways such as in sediments, plant debris, biofilms (Goncalves *et al.*, 2006). Penicillia and aspergilli were recorded as the most common soil fungi present in high numbers in water bodies associated with sediments and biofilms (Goncalves *et al.*, 2006).

Contamination of fish and their eggs with mycotoxin producing fungi was detected early when Kita (1914) isolated *Aspergillus glaucus*, *A. melleus*, *A. ochraceus*, *A. oryzae*, *A. candidus*, *A. versicolor*, *A. sulphureus*, *A. wentii*, *Cladosporium herbarum*, *Mucor racemosus* and *Torula fuliginea* from dried fish. Nofiani *et al.* (2010) isolated *Penicillium corylophilum* as a dominant species from fermented shellfish. *Aspergillus glaucus* was commonly isolated from many species of fishes (Panasenko, 1967). Kikkiwa and Kosuci (1937) isolated *Aspergillus repens* and *A. tuber* from many dried fish samples and proved that these two species actively brake down this type of fish. *Aspergillus*, *Fusarium*, *Paecilomyces*, *Rhizomucor* and *Phoma* have been shown to be implicated in fish diseases (Crow *et al.*, 1995; Wolf and Smith, 1999; Hatai and Egusa, 1979). These fungal species are mostly associated with mycotoxin production (Gottlich *et al.*, 2002; Kelley *et al.*, 2003) which could contaminated fish with mycotoxins either during cultivation or during post-harvesting process (Abdel-Wahhab and Kholif, 2008). Many species especially those belonged to *Aspegillus* are known to produce many types of toxins such as aflatoxins, ochratoxins and sterigmatocystine. These mycotoxins exhibit toxic, mutagenic, teratogenic and carcinogenic effects in human beings (Frisvad *et al.*, 2005; Zinedine *et al.*, 2006; Motalebi *et al.*, 2008). There are many evidences that primary liver carcinoma and other diseases may caused by consuming food or raw materials contaminated with fungi or mycotoxins. There are many report about heat-resistant of aflatoxins, ochratoxin A and sterigmatocystin and their accumulation the organism (Galvano *et al.*, 2005; Jay *et al.*, 2005). Thus presence of the toxins' residue in fish tissues is very dangerous for human even after processing the fishes.

Therefore, the aim of this study was to isolate mycotoxin producing fungi from fishes growing in aquacultures. Detection of different mycotoxins produced by the isolated fungi was carried out.

MATERIALS AND METHODS

Fish samples: Five fish species were collected from fish farms distributed in Delta region, Egypt during the period of June-July, 2010. These species were; *Oreochromis niloticus niloticus* (the Nile tilapia), *Clarias gariepinus* (African catfish), *Tilapia zilli* (Tilapia zilli), *Crenidens crenidens* (Bony bream) and *Liza ramada* (Thinlip Mullet). From each fish species, at least 10 random samples were collected. Each sample contained five individuals collected randomly from each farm. All the collected fishes were preserved in thermostat ice tanks and brought to the laboratory for mycological analysis.

Isolation of fungi from fish samples: Fish samples were washed in running water to remove sediments. The clean samples were rinsed twice in distilled water and dried with sterilized filter papers. Different parts of fishes including skin, fans, gills and intestine were sliced (about 1 cm²). Five pieces were inoculated onto either Sabouraud's agar or Potato Dextrose Agar (PDA) medium (Smith and Onions, 1983). Three plates from each part were used. Media were supplemented with chloramphenicol and streptomycin (66 µg mL⁻¹) to suppress the growth of bacteria. Plates were incubated at 28±1°C for 5 to 7 days during which the growing fungal species were examined and identified.

Identification of fungal species: The growing colonies of different fungal species were examined using ordinary microscope. Microscopic and macroscopic characteristics of the growing cultures were used in identification following the identification keys of Moubasher (1993), Raper and Fennell (1965), Pitt (1979) and Pitt and Hocking (1997).

Screening for mycotoxins production

Cultivation of fungal isolates for toxin production: All fungal species isolated in this study were examined for their capability to produce of their respective mycotoxins. Seven days old culture of each isolate on PDA was used to obtain a spore suspension in 0.2% (v/v) aqueous tween 80 (Smith and Onions, 1983).

Each isolate was inoculated into 250 mL Erlenmeyer flasks containing sterilized 50 mL of glucose-Czapek's liquid medium supplemented with 0.2% yeast extract and 1% peptone. The cultures were incubated at $28\pm 1^\circ\text{C}$ as stationary cultivation for 8 days.

Extraction of mycotoxins: After incubation, the content of each flask (medium+mycelium) was homogenized in a high-speed blender (16000 rpm) with 100 mL chloroform. The chloroform extracts were combined, washed with an equal volume of distilled water, dried over anhydrous sodium sulfate, filtered and concentrated under vacuum and the dry material was transferred to a dram vial with a small amount of chloroform which was evaporated to near dryness. The content of each flask, after decanting the chloroform extract was extract again by 100 mL of 90% aqueous methanol. The aqueous methanol extract was decanted off and re-extracted by another 100 mL methanol. The aqueous methanol extracts were combined, concentrated under vacuum which were extracted again by acetonitrile (3 times), concentrated, transferred to a dram vial and evaporated to near dryness (Zohri and El-Kady, 2003).

Purification of the crude toxins: The dry crude extract was suspended in 50 mL chloroform and applied to silica gel column (200 meshes, MERCK) according to the method described by AOAC (1984). The column was washed with 150 mL n-hexane, followed by 50 mL diethyl ether. Mycotoxins were eluted with 200 mL (95:5, v/v each) of chloroform: Acetone, dichloromethane: Methanol and benzene: Acetone (AOAC, 1984; Jarvis *et al.*, 1986). The elutes were collected and concentrated under vacuum to near dryness. The residue was diluted with chloroform to 1 mL.

Thin layer chromatographic determination of mycotoxins: For determination, thin-layer chromatographic technique adopted by El-Kady and Moubasher (1982) was employed. Glass plates (20×20 cm) were cleaned by washing with detergent, tap water, distilled water and ethanol. Thin layer of silica gel (MERCK, Darmstadt, G.F.R.) of about 0.3 mm thickness were prepared employing an applicator. The plates were air dried, activated in an oven at 110°C for 2 h, then left to cool and stored in desiccators.

Solvent systems: For the purpose of separation of the different mycotoxins, the solvent systems of the following compositions were used, all were of reagents grade: Chloroform: acetone (9:1, v/v) for aflatoxins (Jones, 1972), Chloroform: Methyl alcohol (97:3, v/v) for aflatoxins (Pohland *et al.*, 1970) and Benzene: methanol: Acetic acid (90:2:15, v/v/v) for strigmatocystin as described by Naoi *et al.* (1972).

Application and development: The samples were applied as 0.01 mL solution in chloroform or methanol or mixture of both, using micropipettes. The spots were dried during application with a flow of cold air. The plates were developed in developing tanks of 15×30×30 cm diameter (Zeiss, Jena, G.D.R.) saturated with solvent vapor. Each substance was chromatographed in two series in all the solvent systems. When the front of the systems reached a height of about 15 cm above the origin, the development was interrupted, the chromatogram was dried in air and then detection was carried out.

Determination and reagents: The developed plates were detected before and after spraying with the different reagents under short wave (254 nm) and long wave (354 nm) ultra violet irradiation (UV IS, Desage, Heidelberg, G.F.R.). Mycotoxins were identified by comparison with appropriate reference standards.

RESULT AND DISCUSSION

Twenty-one fungal species were isolated from five fish species collected from aquacultures (Table 1). The highest number of species was isolated from *C. garipinus* (15 species), followed by *O. niloticus* (14 species). Relatively low number of species was isolated from *L. ramada* and *T. zilli* (10 and 9 species, respectively). However, the lowest number of species was recorded from *C. crenidens* (6 species). *Paecilomyces lilacinus*, *P. variotii* and *Ph. herbarum* were the most common species, where they were isolated from the five fishes. The highest value of isolation

Table 1: Percentage of isolation of different fungal species from five fishes on PDA medium at 28±1°C

Fungal species	<i>Oreochromis niloticus</i> (n = 16)	<i>Tilapia zilli</i> (n = 12)	<i>Liza ramada</i> (n = 12)	<i>Clarias garipinus</i> (n = 12)	<i>Crenidens crenidens</i> (n = 12)
<i>Aspergillus carneus</i>	-	-	50.0	100.0	100.0
<i>A. clavatus</i>	-	-	-	33.3	-
<i>A. flavus</i>	37.5	100.0	83.3	-	-
<i>A. niger</i>	100.0	50.0	-	83.3	-
<i>A. ochraceus</i>	25.0	33.3	50.0	-	33.3
<i>A. parasiticus</i>	37.5	50.0	-	50.0	-
<i>A. sydowii</i>	-	50.0	-	-	-
<i>A. terreus</i>	-	-	-	-	83.3
<i>A. ustus</i>	-	-	33.3	66.7	-
<i>A. versicolor</i>	-	16.7	33.3	83.3	-
<i>Cladosporium cladosporioides</i>	-	-	66.7	83.3	-
<i>Mucor heimalis</i>	12.5	-	-	-	-
<i>Paecilomyces lilacinus</i>	50.0	83.3	50.0	66.7	50.0
<i>P. variotii</i>	37.5	50.0	33.3	66.7	100.0
<i>Penicillium brevicompactum</i>	37.5	-	-	50.0	-
<i>P. chrysogenum</i>	62.5	-	-	-	-
<i>P. corylophilum</i>	50.0	-	-	66.7	-
<i>P. stekii</i>	37.5	-	50.0	83.3	-
<i>Phoma herbarum</i>	12.5	50.0	66.7	100.0	66.7
<i>Rhizopus stolonifer</i>	37.5	-	-	50.0	-
<i>Trichoderma viride</i>	37.5	-	-	33.3	-
Total number of species	14	9	10	15	6

percentage of *P. lilacinus* was recorded as 83.3% from *T. zilli* followed by *C. crenidens* (66.7%), however its lowest values were obtained from the other three fishes as 50%. *P. variotii* recorded its maximum value of isolation on *C. crenidens* (100%), however on *L. ramada* it had the lowest value as 33.3%. On *C. garipinus*, *Ph. herbarum* was isolated in the highest value (100%), however its lowest value was recorded on *O. niloticus* (12.5). *Aspegillus clavatus* was isolated only from *C. garipinus*. *A. terreus* was recorded only from *C. crenidens*, however *Penicillium chrysogenum* was recovered only from *O. niloticus*. The wide variation of fungal species encountered for *C. garipinus* (african catfish) could be because unique mode of this fish where it usually buried itself in the mud and debris that increase the opportunity for contamination with terrestrial fungal spores. Fish contamination with the terrestrial fungi either as saprophytes or parasites was previously reported. Among these fungi *Aspergillus*, *Paecilomyces* and *Phoma* were reported as causal pathogen of fish diseases (Crow *et al.*, 1995; Wolf and Smith, 1999).

Figure 1 shows that 15 fungal species were isolated from *C. garipinus* in various degrees of abundance. *A. carneus* and *Ph. herbarum* were the most frequent species, where their total count represented 9.84% of the gross total count (for each). The total count of *A. niger*, *A. versicolor*, *C. cladosporioides* and *Penicillium stekii* was 8.2% (for each). *P. parasiticus*, *P. brevicompactum* and *R. stolonifer* were isolated in low count (4.92% for each). The lowest count was detected in case of *A. clavatus* and *Trichoderma viride* (3.2% for each). Figure 2 showed that among the fourteen species isolated from *Oreochromis niloticus*, both *A. niger* and *A. flavus* were the dominant species with total count of 16 and 14%, respectively of the gross total count. *P. chrysogenum* followed these two species in the rank with 10% of the total count of the all species. The count of the rest of the isolated fungal species was fluctuated from 2 to 8% of the gross total count. Data in Fig. 3 represented the total count of isolated fungal species from *Liza ramada*. *A. flavus* occupied the first order where it comprised 16.13% of the total count followed by *Cladosporium cladosporioides* (12.9%) and *Phoma herbarum* (12.9%). *A. ochraceus*, *P. lilacinus* and *P. stekii* were isolated in relatively low count (9.7% for each), however both *A. versicolor* and *P. varotii* comprised the lowest detected total count (6.5% for each). Figure 4 contains the total count of isolated fungal specie from *Tilapia zilli*. It is clear that the most common species was *A. flavus* (21.43%). *Phoma herbarum*

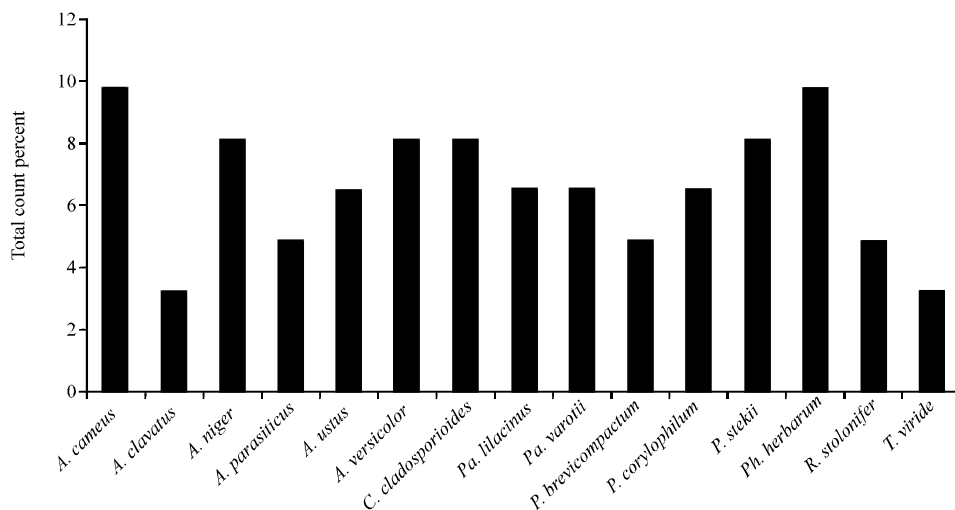


Fig. 1: Total count percent of isolated species from *Clarias garipinus* on PDA at 28±1°C

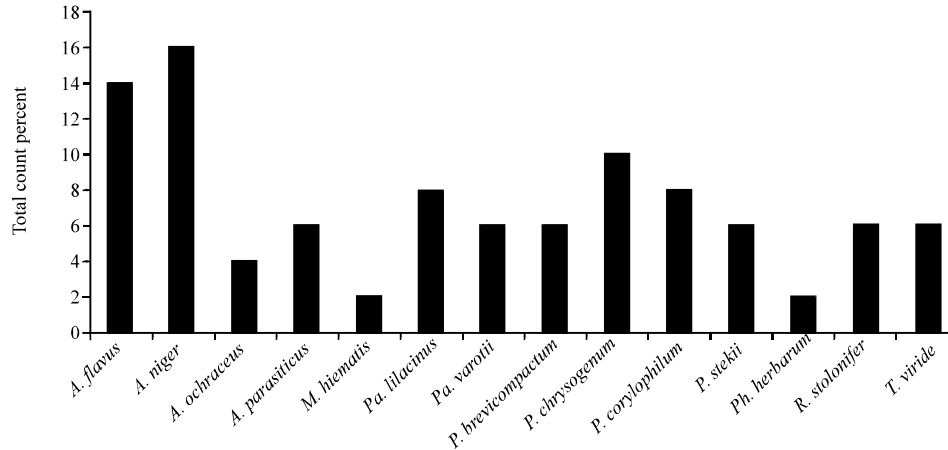


Fig. 2: Total count percent of isolated species from *Oreochromis niloticus niloticus* on PDA at 28±1°C

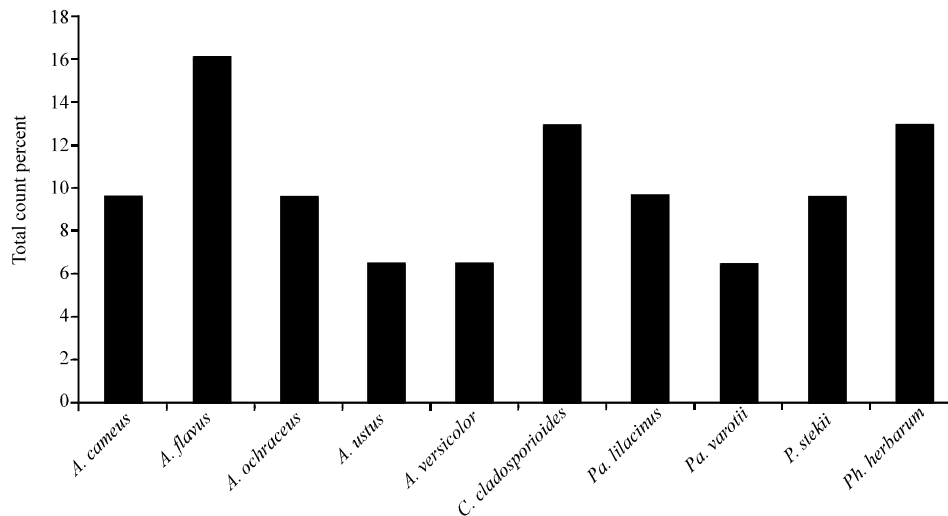


Fig. 3: Total count percent of isolated species from *Liza ramada* on PDA at 28±1°C

came in the second order and it counted 17.9% of the gross total count. The count of five species namely; *A. niger*, *A. parasiticus*, *A. sydowii*, *P. varotii* and *Ph. herbarum* was 10.7% for each. *A. versicolor* emerged the lowest count (3.57). In Fig. 5, the relative total count of six fungal species isolated from *Crenidens crenidens* was represented. *A. carneus* and *P. varotii* were the most common species where they counted 46% of the gross total count (23% for each). *A. terreus* occupied the second order after these two species, it counted 19.2% of the total count. *Phoma herbarum* and *P. lilacinus* counted 15.4% and 11.5%, respectively. *A. ochraceus* represented the only 7.7% of the gross total count of the isolated fungi. Wheeler *et al.* (1986) isolated many fungi from dried salted fish from Indonesia. These species were *Eurotium rubrum* (35%), *E. repens* (26%), *E. amstelodami* (22%), *E. chevalieri* (18%), *Aspergillus niger* (37%), *A. flavus* (27%), *A. sydowii* (23%), *A. penicilloides* (16%) and *A. wentii* (15%). In agreement with our results, Ali *et al.*, 2011) isolated *Aspergillus flavus*, *A. niger*, *A. parasiticus*, *A. versicolor*, *Mucor heimalis*, *Paecilomyces lilacinus*, *Pa. varotii*, *Phoma herbarum*, *Rhizopus stolonifer* and *Trichoderma*

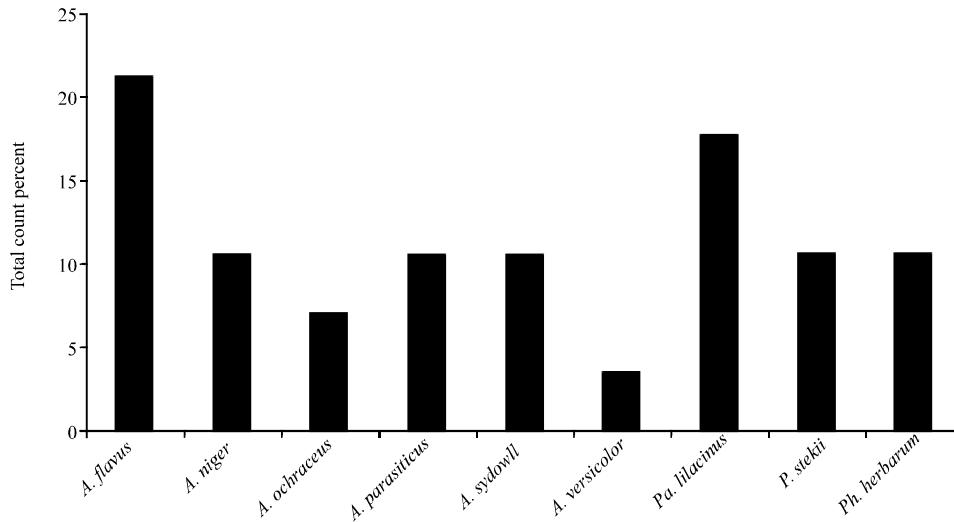


Fig. 4: Total count percent of isolated species from *Tilapia zilli* on PDA at 28±1°C

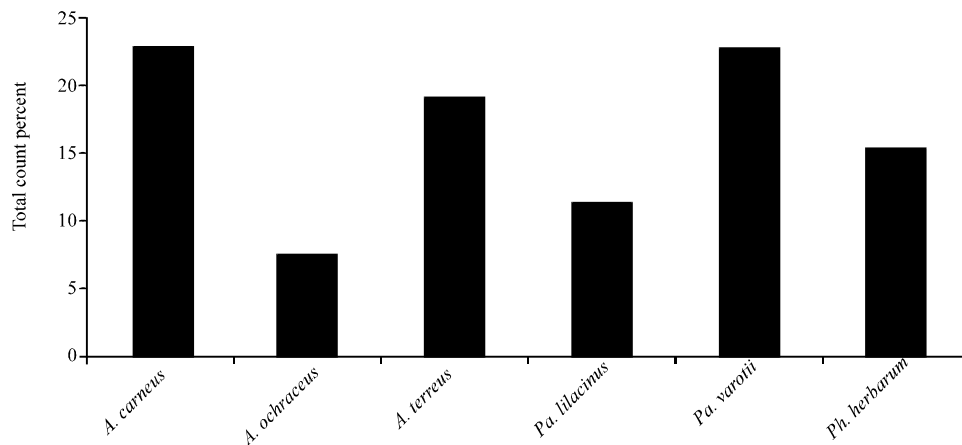


Fig. 5: Total count percent of isolated species from *Crenidens crenidens* on PDA at 28±1°C

hamatum from *Oreochromis niloticus niloticus* and *Clarias gariepinus* in different frequencies. They reported that the most prevalent soil pathogenic fungi isolated from diseased Nile tilapia were *Paecilomyces lilacinus* and *Phoma herbarum*. Adebayo-Tayo *et al.* (2008) estimated the mycoflora and aflatoxin contamination of smoked dried different fishes and reported that twelve different fungi were found associated with smoked dried fishes samples sold in three different markets. The associated fungi were *Aspergillus flavus*, *A. terreus*, *Aspergillus fumigatus*, *Absidia* sp., *Rhizopus* sp., *A. niger*, *Mucor* sp., *Cladosporium* sp, *Penicillium italiculum*, *Penicillium viridatus* *Candida tropicalis* and *Fusarium moniliforme*, *Aspergillus flavus* and *A. terreus* had the highest rate of occurrence among the isolated fungi. These findings greatly support our results.

Table 2 showed that nine fungal species isolated from the studied fish species were able to produce mycotoxins. *A. flavus* was able to produce four toxins including Aflatoxin B1, B2 and G1 in addition to stregmatocystin in synthetic culture media. *A. parasiticus* produced two mycotoxins; Aflatoxin G1 and stregmatocystin. The other seven species produced only one mycotoxin.

Table 2: Mycotoxin production by fungal species isolated from different fish species

Fungal species	Aflatoxin			Sterigmatocystin	Ochratoxin	Patulin	T2 -toxin
	B1	B2	G1				
<i>Aspergillus clavatus</i>	-	-	-	-	-	+	-
<i>A. flavus</i>	+	+	+	+	-	-	-
<i>A. ochraceus</i>	-	-	-	-	+	-	-
<i>A. parasiticus</i>	-	-	+	+	-	-	-
<i>A. sydowii</i>	-	-	-	+	-	-	-
<i>A. terreus</i>	-	-	-	-	-	+	-
<i>A. versicolor</i>	-	-	-	+	-	-	-
<i>Penicillium chrysogenum</i>	-	-	-	-	+	-	-
<i>Trichoderma viride</i>	-	-	-	-	-	-	+

+: Presence, -: Absence

Sterigmatocystin was produced by both *A. sydowii* and *A. versicolor*. Ochratoxin was detected from *A. ochraceus* and *P. chrysogenum*. Patulin was detected in the cultures of both *A. clavatus* and *A. terreus*. T2-toxin was only produced by *T. viride*. With agreement of these results, Jonsyn and Lahai (1992) mentioned that 20 samples of Bonga, a smoke-dried fish, obtained from homes and markets in Njala, Sierra Leone were contaminated with four *Aspergillus* spp. *A. flavus*, *A. ochraceus*, *A. tamarii* and *A. niger*. Varying amounts of aflatoxins B1, G1 and G2 were detected in the moldy fish.

All of these fungal species have been previously reported to have the ability to produce mycotoxins (Bugno *et al.*, 2006; Reddy *et al.*, 2010; Iheshiulor *et al.*, 2011). According to Pohland and Wood (1997), 70-80% of the penicillia are potential mycotoxins producer (citrinin, patulin, cyclopiazonic acid and penitern).

Contamination of fish with mycotoxin-producing fungi could lead to accumulation of these toxins in fish tissues. The risk for mycotoxins contamination may be occurred as a result of using the contaminated fish tissues, especially in great quantities. Aflatoxins, ochratoxin A and sterigmatocystin proved resistant to heat and have an ability to accumulate in the organism (Galvano *et al.*, 2005). Even products stored at low temperatures are vulnerable to some fungi (Durakovic *et al.*, 1989). Therefore processing of fish does not exclude or minimize the presence of mycotoxins in fish tissues. In this context, Adebayo-Tayo *et al.* (2008) reported that aflatoxin B and G was detected in all smoked dried fishes samples contaminated with *Aspergillus flavus*. These results supports our hypothesis that the contaminated fishes with fungi contain mycotoxin residues that affect the public health. Aflatoxins are highly carcinogenic, causing hepatoma (cancer of the liver) and have also been associated with acute hepatitis in man, mostly the developing world (Eaton and Groopman, 1994; Krogh, 1992; Prasad, 1992). Contamination of rats with sterigmatocystin, a major secondary metabolic of *Aspergillus*, leads to significant drop in liver antioxidants including vitamin E (Sivakumar *et al.*, 2001). Goldbatt (1983) reported that aflatoxins are occurred in the human diet and this could pass from feed to milk. Prolong intake of this metabolites may constitute a health hazard (Akande *et al.*, 2006).

CONCLUSION

The main conclusion of this study is the confirmation of the infection of aquacultures' fishes with mycotoxin-producing fungi. The most detected mycotoxins were aflatoxin B1, B2, G1,

stregmatocystin ochratoxin and T2-toxin. The study recommends periodical examination of fishes grown in these aquacultures to ensure that they are devoid of fungal contamination to conserve the public health. It is therefore important that both the owners of the fish's farms and governmental authorities should adapt a better method to prevent fungal contamination of fishes during growing periods and during handling processing and transportation.

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