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Study of Mycoflora, Aflatoxigenic Fungi and Aflatoxin in Fish Feeds.

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ABSTRACT

Sample of fish feeds were collected from Mumbai, agar-plate methods were used for isolation of mycoflora associated with collected samples. All isolates of Aspergi1lus flavus obtained from collected samples were screened for their aflatoxigenic potential in SMKY liquid medium. In all seventeen different fungi were isolated from fish feeds. Aspergi1lus flavus, *A. niger, A. ochraceous, Aspergi1lus sp., Fusarium sp.* and *Penicil1ium sp.* were stand out common seed infesting fungi, *A. flavus* was dominant in all fungi and its 76% strains were aflatoxigenic. Highest percentage of aflatoxigenic fungi was recorded in Feed Ingredients (86.2%). Analysis of fish feeds for its natural aflatoxin contamination revealed that 26.67% samples were naturally contaminated with aflatoxin. Maximum concentration of aflatoxin $B_1$was detected in Local fish feed (93.67 ppb) followed by Feed Ingredients (92.89 ppb) and Commercial fish feed (84.38 ppb). Oil seeds contaminated with aflatoxin has poses a potential threat for the life of aquaculture animals.

Keywords – fish feeds, Mycoflora, Aspergillus flavus, aflatoxin.

INTRODUCTION

Aflatoxicosis is a disease that can affect many species of fish, and results when feed contaminated with aflatoxins is eaten by the fish (Ashley, 1970). Aflatoxins are one of the most potent toxic substances that occur naturally. These are a group of closely related mycotoxins produced by fungal species such as Aspergillus flavus and Aspergillus parasiticus. Which commonly grow on human foods and animal feeds(Dwarkanath *et al.*, 1969; Kolhe, (2016); Nagarajan and Bhat,1973; Basappa *et al.*, 1977; Kolhe, *et al.*,1994; Verma *et al.*, 1996, Kolhe and Chaudhari, 2011, Chaudhari and Kolhe, 2017). Oilseed crops are primarily soybeans, sunflower seed, canola, rapeseed, safflower, flaxseed, mustard seed, peanuts and cottonseed, Wheat and Maize. After extraction of the oil the residue is a valuable source of protein, especially for animal feeding stuffs, as in oil-seed cake or press cake.

Four major aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) are direct contaminants of grains and finished feeds. Factors that increase the production of aflatoxins in feeds include environmental temperatures above 27°C (80°F), humidity levels greater than 62%, and moisture levels in the feed above 14%. The extent of contamination will vary with geographic location, feed storage practices and processing methods. Improper storage is one of the most important factors favoring the growth of aflatoxin-producing molds, and it is a major element that can be controlled by the fish producer. It is necessary to know the mycoflora, incidence of aflatoxigenic fungi and aflatoxin contamination. The present investigation is an attempt in that direction.

MATERIALS AND METHOD

Samples of fish feeds (Feed Ingredients, fish feed Local, Commercial fish feed) were randomly collected from different Centre of Mumbai, Maharashtra during February - May 2018. To minimize the loss of water content, the samples were collected in a sterile polyethylene bag and sealed, transferred without delay to the laboratory, kept at 4ºC until analysis. Isolation of mycoflora was done by agar plate methods using peptone, glucose, rose bengal agar medium containing streptomycin. (Booth,1971). Fungal colonies formed were identified and percent incidence of each fungus was calculated.

The isolates of Aspergillus flavus were screened for their aflatoxin producing potentials in SMKY liquid medium (Diener and Davis, 1966). Ten days old culture filtrates were extracted with chloroform (v/v) and qualitatively analyzed for different types of aflatoxins on TLC plates (Reddy *et al.*, 1970).

For analysis of aflatoxin contamination in seeds. The oil seeds samples were examined under U.V. light for BGYF test (Fennell *et al.*,1973). Powdered seeds were extracted with methanol: water (6:4 v/v) and sodium chloride (Anon, 1975). The aqueous methanolic extract was defatted using n-hexane followed by its extraction for aflatoxin with chloroform which was processed for qualitative analysis of aflatoxin on TLC plates (Reddy *et al.*, 1970).

The TLC plates were air-dried and observed under long-wave UV light (360nm) for aflatoxins (B_1, B_2 ,G_1&G_2). The aflatoxins were also chemically confirmed by spraying trifluoroacetic acid and 25% sulfuric acid. Each spot was scraped separately, dissolved in chilled methanol and subjected to spectrophotometric measurement at 360 nm using a temperature controlled using shimadzu UV160A Spectrophotometer (Nabney and Nesbitt, 1965).

RESULT AND DISCUSSION

Isolation of mycoflora: Table 1 records the fungi (in *%)* isolated from fish feeds. It is obvious that Aspergilli out numbered other genera, viz. Aureobasidium, Alternaria, Cladosporium, Curvularia, Fusarium, Mucor, Penicilium and *Rhizopus sp. Aspergillus flavus* was found dominant on all types of fiosh feeds. Depending on percent incidence different fish feed can be arranged in the following decreasing sequences.

Feed Ingredients > Local fish feed > Commercial fish feed.

Aspergillus flavus, *A. niger* and *A. ochraceus* emerge as the most common infesting fungi of the test oil seeds. *Aspergillus candidus, A. terreus* and *A. parasiticuson* other hand, were encountered only Feed Ingredients. After Aspergilli, the most common infestant was *Altenaria sp., Fusarium, sp.* and *Penicillium sp.* (isolated from all fish feed), followed by *Cladosporium, Curvularia, Mucor* and *Rhizopus sp.* (isolated from Feed Ingredients and Commercial fish feed) and *Aureobasidium pullulans* (isolated from Local fish feed).

Aflatoxigenic potentials of *Aspergillus flavus* isolates obtained from fish feeds:

Altogether 75 isolates of Aspergi1lus flavus obtained from fish feeds and screened for their aflatoxin producing potentials in SMKY liquid medium (table 2), only 57 isolates were aflatoxin producers. The incidence of toxigenic isolates varied with the commodities from which they were isolated. Depending upon the presence of toxigenic A. flavus isolates of fish feeds could be arranged in the following decreasing sequence:

Feed Ingredients > Local fish feed > Commercial fish feed.

Toxigenic isolates of A. flavus obtained from fish feeds, elaborated different components of aflatoxins in liquid medium. Aflatoxin B_1 was elaborated by all toxigenic isolates of A. flavus (57).

Table 1: Mycoflora associated with fish feed and their percentage incidence

Mycoflora	fish feed		
	Feed Ingredients	Local fish feed	Commercial fish feed
Aspergillus flavus	58	55	46
A. niger	11	20	20
A. candidus	1	------	-----
A. terreus	2	--------	-----
A. ocharaceus	10	5	
A. parasiticus	2	__	
Aspergillus sp.	3	6	10
Alternaria sp.	1	1	2
Aureobasidum pullulans	__	2	----
Cladosporium sp.	1	-------	1
Curvularia sp.	2	-------	1
Fusarium sp.	4	7	10
Mucor sp.	2	-------	2
PeniciIlium sp.	2	4	5
Rhizopus sp.	1	------	3

Table 2: Aflatoxin producing potentials of Aspergi1lus flavus obtained from fish feeds.

fish feed	Number of isolates		Number of Isolates producing aflatoxin				Amount of aflatoxin B_1(mean) ppm
	Screened	Toxigenic (percent)	B_1	B_1+B_2	$B_1+B_2+G_1$	$B_1+B_2+G_1+G_2$	
Feed Ingredients	29	25(86.2)	13	7	3	2	4.38
Local fish feed	27	22(81.5)	8	10	3	1	1.92
Commercial fish feed	19	10(52.6)	7	2	1	--	1.63
Total	75	57(76)	28	19	7	3	

Table 3: Aflatoxin contamination in fish feeds.

Sample of fish feeds	Number of samples							Aflatoxin B_1 Concentration (mean) ppb
	Screened for aflatoxin contamination	Positive BGYF test	Positive to aflatoxin contamination (Percent)	Samples Positive For				
				B_1	B_1+B_2	$B_1+B_2+G_1$	$B_1+B_2+G_1+G_2$	
Feed Ingredients	20	15	7 (35.0)	2	4	1	1	92.89
Local fish feed	20	17	5 (25.0)	4	3	1	1	93.67
Commercial fish feed	20	10	4 (20.0)	1	2	-	-	84.38
Total	60	42	16 (26.67)	7	9	2	2	---

None of the isolates elaborated aflatoxin B_2, G_1 or G_2 in absence of AFB_1; 28 isolates could produce AFB_1 only, 19 isolates were capable of elaborating both AFB_1 and B_2 while 7 isolates were able to produce AFG_1along with AFB_1 and B_2. Only three isolates could produce all four types of aflatoxins (B_1, B_2, G_1 and G_2). Table 2 also reveals that AFB_1 producing potentials of toxigenic A. flavus isolates varied with the type of fish feeds; while isolates from Feed Ingredients were highly aflatoxigenic, isolates recorded from Commercial fish

feed were least. Raper and fennell (1965) also reported that all A. flavus isolates are not toxigenic. In 1965, Hiscocks noted that some isolates of A. flavus produced either B or G toxins, but majority of them produced both toxins. None of the isolates produced B_2, G_1 and G_2 in absence of B_1 (Lillehoj *et al*., 1977). It has been suggested that toxigenic nature of the isolates is possibly governed by their genetic makeup (Ciegler, 1977). Mold growth and toxin formation require a moisture content of the substrate greater than 14 % and a temperature of approximately 25 %. Reduced oxygen content diminishes aflatoxin formation (Diener *et al*., 1987).

Aflatoxin contamination in fish feeds.:

Out of All total 60 samples screened for their aflatoxin contamination, only 42 samples were found positive to BGYF test (table 3). But extraction studies revealed that only 16 samples were positive for aflatoxins. Result shows that about 35% Feed Ingredients samples were contaminated with aflatoxin followed by Local fish feed (25%) and Commercial fish feed (20%). In case of Feed Ingredients and Local fish feed all the four types of aflatoxins were identified; AFB_1+B_2 were present in Commercial fish feed. Based on the concentrations of AFB_1 (ppb) recorded, samples of fish feeds could be arranged in the following decreasing orders:

Local fish feed > Feed Ingredients > Commercial fish feed.

Indian climatic conditions coupled with socio-economic backwardness offer excellent conditions for mycotoxin production. Hot and humid climate, which is ideal for mould growth, is prevalent in most parts or India, particularly during the monsoon season. The consumption of mycotoxin contaminated food often becomes indispensable due to acute food shortage and poverty.

Fish feed is the major cost item in the aquaculture industry and constitutes 40–50% of the total production costs in intensive culture systems (Enyidi *et al*., 2017). Feed cost may be reduced by incorporating vegetable oil, increasing levels of plant ingredients, and reduction in the level of costly fishmeal (Enyidi *et al*., 2017). However, plant-based ingredients have been associated with contaminants produced by fungi during the initial stages of crop production (Embaby *et al*., 2015). During processing, feed can be contaminated with fungal spores, particularly when grains are ground and the feed pelleted (Embaby *et al*., 2015). Feed storage practices and processing methods, environmental temperatures >27 °C, humidity levels >62%, and moisture levels in the feed >14% are some of the factors that can increase fungal growth in feed, and this may result in mycotoxin production (Mahfouz and Sherif, 2015). Some isolates that produce aflatoxin under cultural conditions, fail to do so under natural conditions. This could be due to unfavourable and changing conditions and the effect of interaction with other microorganisms. Besides, genetical factors might also be responsible for the variations in aflatoxin production by different strains of A. flavus (Maggon *et al*., 1969; Ciegler, 1977). Damage of cereals by insects such as weevils and physical damage can greatly increase Aspergillus infection and the levels of aflatoxins. Protein supplements such as cotton seed cakes, sunflower cakes, fish meal and other oil seed by products which are often poorly stored are the primary source of the mould found in homemade dairy concentrates on small hold farms (Lunyasunya *et al*., 2005). Exposing fish to mycotoxigenic fungi would subsequently reduce their growth rate, damage the liver, reduce immune responsiveness, increase mortality, and lead to a steady and gradual decline in quality of reared fish stock, posing serious challenges to aquaculture development (Fallah *et al*., 2014). Therefore, need for regular monitoring of aflatoxin contamination in fish feeds for quality control, and to develop method which can reduce the chances of aflatoxin production during storage and transport.

CONCLUSION

The present study indicates high level of contamination of fish feed with different fungal species but specially the *Aspergillus flavus*. were dominant. The co- occurrence of aflatoxin B_1 present a health risk because of their synergistic and /or additive effect. Aflatoxin can be carried over to human food of animal origin; human exposure to aflatoxin may cause health threats. The study show that feed ingredient are important vehicle for contaminating finished fish feed as they may be heavily contaminated by aflatoxin. Feed manufactures should monitor feed routinely and appropriate aflatoxin absorbents should be selected to reduce contamination in fish feed.

Conflicts of Interest: The authors declare no conflict of interest.

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