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Qualitative detection of aflatoxins and aflatoxigenic fungi in wheat flour from different regions of Egypt

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Abstract: *The presence of fungi and aflatoxins in wheat flour samples collected from five governorates (El-Minia, Assiut, Sohag, Qena and Aswan) in Egypt were evaluated over three months. Samples were assessed for moisture content, fungal infestation and aflatoxin contamination. During the three months, moisture contents ranged from 9.31 % and 12.4%.* Aspergillus flavus *was the most predominant fungal species, the maximum cfu/g was* $8.3x10^2$. *Other fungal genera and species isolated including* A. niger, A. tamarii, A. clavatus, A. nidulans, Penicillium *spp.,* Mucor *spp.,* Fusarium *spp.,* Alternaria *spp.,* Rhizopus stolonifer, Cladosporium cladosporioides *and yeasts. Cultural and analytical methods (coconut based medium test, ammonia vapour test and thin layer chromatography (TLC) assay) were used to analyze the potential aflatoxins production by strains isolated from wheat flour and aflatoxins in wheat flour samples.*
Keyword: Aspergillus flavus*, aflatoxins, wheat flour, cultural and analytical methods.*

I. Introduction

Throughout the last decade, food safety and consumer protection have gained increasing importance. Hence, the proof of authenticity, detection of fraud and determination of residues and contaminants (e.g. drugs, pesticides or mycotoxins) are a focus of current interest (**Koch *et al.,* 2009**).

Wheat (*Triticum aestivum* L.), family Poaceae is one of the most important food of about two billion people (36% of the world population). Worldwide, wheat provides nearly 55% of the carbohydrates and 20% of the food calories consumed globally in diets (**Embaby *et al.,* 2012**).

Wheat represents a very suitable substrate for various phytopathogenic and sabrophytic microorganisms (**Tatjana *et al.,* 2005**). Cereals and cereal products represent a significant food resource for the world's population. Various microbial contaminants can grow on cereal grains and finished products held under unsuitable storage conditions (**Deibel and Swanson, 2001**). Flour is considered as a microbiologically safe end product as it has a low water activity commodity. Moisture is an important significant parameter in flour that affects shelf life and proliferation of spoilage microorganisms (**ICMSF, 1998**).

When the water content exceeds the critical level for wheat flour (13%-15%) moulds start growing (**Jay, 1996**). This situation illustrates how some physico-chemical parameters could affect microbiological parameters. Mould growth in flour is known to decrease its quality significantly (**Aydin *et al.,* 2009**).

Healthy seeds play an important role for successful cultivation and increasing crop yield (**Wiese, 1984**). Several seed-borne pathogens are known to be associated with wheat seed which are responsible for the deterioration of seeds quality during storage (**Doohan *et al.,* 2003**).

Representatives of *Aspergillus, Penicillium, Fusarium* and *Alternaria* genera are more responsible for producing mycotoxins. Microscopic fungi can produce these poisonous substances not only during the vegetation on grain crops, but also during storage time (**Davis and Diener, 1987; Bayman *et al.,* 2002; Drusch and Aumann, 2005; Burkin *et al.,* 2008**). The Food and Agriculture Organization (FAO) estimates that mycotoxins contaminate about 25% of agricultural crops globally (**WHO, 2006**). Mycotoxins may be produced during any stage of production; harvest, transportation, storage or processing (**CAST 2003; Murphy *et al.,* 2006a,b**). In a previous study (**Marija *et al.,* 2004**), they detected a higher contribution of major mycotoxigenic fungi in flour, making the product more susceptible to the accumulation of mycotoxins.

Aflatoxins and ochratoxin A (OTA) are the most important naturally occurring mycotoxins in agricultural products. Aflatoxins are produced by several species classified within the section Flavi of subgenus Circumdati as *Aspergillus* (*A. bombycis, A. flavus, A. nomius, A. ochraceoroseus, A. parasiticus* and *A. psudotamarii*), *Emericella astellata* and *Peteromyces alliaceus* (**Samson *et al*., 2006, Varga and Samson, 2008**). Not all strains of *Aspergillus* have the ability to produce mycotoxins, therefore, it is important to screen them for toxin production abilities. **Davis et al. (1987)** discovered a method using coconut agar medium (CAM) for detection of aflatoxins. In this method, aflatoxigenic isolates exhibited blue fluorescence on the reverse side of the colony under UV light.

Cultural methods for detection of aflatoxigenic and non-aflatoxigenic *Aspergillus* using ammonia vapour **(Saito and Machida, 1999; Kumar *et al.*, 2007)** and cyclodextrine derivatives **(Fente *et al.*, 2001)** as indicators on *Aspergillus* colonies grown on yeast extract sucrose (YES) agar have been described. In these methods, aflatoxigenic strains produced a pink color against ammonium vapour and green-blue fluorescence under UV light on the reverse side of colonies, respectively.

Analytical methods carried out by **Filtenborg et al. (1983)** for detecting aflatoxins in pure culture is the agar plug sampling thin layer chromatography (TLC) method. TLC is one of the most widely used separation techniques in mycotoxin analysis is well suited for aflatoxins which fluoresce strongly under long-wave UV light. Many analytical and immunological methods are available for assessment of aflatoxins such as high performance liquid chromatography (HPLC) and enzyme linked immune-sorbent assay (ELISA) **(Yazdani *et al.*, 2010)**.

II. Materials and Methods

1. Collection of wheat flour samples:

Thirty samples of wheat flour were collected from bakeries at five governorates (El-Minia, Assiut, Sohag, Qena and Aswan) in Egypt over three months.

2. Determination of moisture content:

The moisture content of wheat flour samples was determined by oven-drying method according to techniques of the **International Seed Testing Association (1966)**.

3. Mycobiota analysis:

This was made by using "Dilution-Plate Method" for the quantitative determination of fungi as described by **Moubasher et al. (1972)**. Czapek's agar medium was used for isolation and purification of fungi.

4. Aflatoxins production ability:

In each sample, isolates of *Aspergillus flavus* were screened for their potential of aflatoxins production.

4.1 *Coconut based medium test*

Coconut milk agar (CMA) medium was prepared based on **Davis et al. (1987) and Dyer and Mc Cammon (1994)**. The plates were inoculated with spores and incubated at 28°C.The presence or absence of a fluorescence ring in the agar surrounding the colonies under UV light after 7 days incubation was noted and the results were recorded as positive or negative.

4.2 *Ammonia vapour test*

Aspergillus isolates were grown on YES agar (2% yeast extract, 15% sucrose and 1.5% agar) and incubated at 28°C **(Saito and Machida, 1999; Kumar *et al.*, 2007)**. After 7 days, the plates were inverted over 2 ml of ammonium hydroxide. After ten minutes, the undersides of aflatoxigenic isolates turned into pink to red color.

4.3 Aspergillus *differential medium (ADM)*

Aspergillus isolates were subjected to ADM as described by **Bothast and Fennell (1974)**.

4.4 *Bioassay methods for mycotoxins (Brine shrimp test)*

The immature stage (nauplii) of brine shrimp (*Artemia salina* L.) was used for mycotoxins bioassay. Larvae of shrimp are suitable for rapid assay of extremely small quantities of mycotoxins **(El-Maghraby, 1996; Ding *et al.*, 2008)**.

4.5 *Thin layer chromatography (TLC) Assay*

Aspergillus isolates were grown on yeast extract sucrose agar (YES), the culture plates were incubated at 28°C for 7 days and examined for aflatoxins using the TLC analysis as reported by **Samson et al. (2002)** and **Hans and Walter (1986)** with some modifications. Agar plugs were prepared by cutting the fungal colony to a diameter of 5 mm. The plugs were immersed in 2 mL of chloroform for extraction of toxin from fungi. The extracts (10 µL) were spotted on TLC plates. Subsequently, 10 µL of mycotoxin standard solution of aflatoxins B1 and G1 (Sigma-Aldrich, Dorset, UK) were used as reference standards and spotted along with the fungal samples extract. The plates were developed in a solvent tank containing chloroform : toluene : acetone (75:15:10) for 1h and viewed under long wave UV light (365 nm). Sample extracts were compared with reference standards spots.

5. Detection of aflatoxins in flour samples

One hundred grams of each flour sample were defatted with n-hexane then extracted with chloroform. The extracts were dried over anhydrous sodium sulphate, filtered then concentrated under vacuum to near dryness **(Youssef, 2008)**. Extracts were examined for aflatoxins using the TLC technique as reported by **Samson et al. (2002)** and **Hans and Walter (1986)** with some modifications.

III. Results

The moisture contents of great majority, the mean moisture contents of thirty wheat flour samples were fluctuated between 9.31 % and 12.4%.

From thirty samples used for fungal isolation, all samples showed fungal contamination. Some samples were contaminated with both *Aspergillus* section *Flavi* and section *Nigri*, from which only those that looked close to Flavi section were selected for subsequent purifications steps. *Aspergillus flavus* was the most predominant fungal species. The maximum cfu⁄g was $8.3x10^2$. Many samples showed contamination with *Penicillium* spp. (about 69.76% of total counts) "Table 1". These plates were used for further *Aspergillus* isolation and purifications. Sixty eight isolates of *A. flavus* were identified and undergone qualitative assays for aflatoxins production. CMA and YES Petri dishes inoculated with purified fungal isolates were screened for their aflatoxins production and TLC assay "Table 2". Results showed that 9 isolates give fluorescence within CMA test "Fig. 1a&1b" while 8 isolates are positive ammonia vapor test "Fig. 2a&2b" and 2 isolate extracts are TLC positive "Table 2". All isolates exhibited reverse pigmentation on ADM, but showed no fluorescence under UV radiation. Detection of aflatoxins in wheat flour samples using TLC detection showed no results.

IV. Discussion

The presence of fungi and aflatoxins in wheat flour samples collected from five governorates (El-Minia, Assiut, Sohag, Qena and Aswan) in Egypt were evaluated over three months. Samples were assessed for moisture content, fungal infestation and aflatoxin contamination. During the three months, moisture contents ranged from 9.31 % and 12.4%. *Aspergillus flavus* was the most predominant fungal species, the maximum cfu⁄g was $8.3x10^2$.

Moisture is an important parameter in flour that has significant effects on shelf life and growth of microbial growth **(ICMSF 1998)**. Changes in dry flour moisture contents of 1% or 2% have been reported to be sufficient for growth and toxin contamination **(Eyles *et al.,* 1989)**.

Rezazadeh et al. (2013) isolated *Aspergillus* (*A. niger*, *A. fumigatus*, *A. flavus*, *A. glaucus*, *A. triticum*), *Acremonium*, *Alternaria*, *Fusarium*, *Mucor*, *Penicillium* and *Cladosporium* spp. from 89 flour samples; but the most common fungi in cultured samples were as follows, respectively: *A. niger*, *Fusarium*, *Acremonium* and *A. fumigates*. The mean mould counts in 142 wheat flour samples from 7 locations studied were 7.4×10^1 to 1.8×10^4 cfu g^{-1} **(Aydin *et al.,* 2009)**.

Cultural and analytical methods (coconut based medium test, ammonia vapour test and thin layer chromatography (TLC) assay) were used to analyze the potential aflatoxins production by strains isolated from wheat flour and aflatoxins in wheat flour samples.

For the detection and determination of aflatoxins, various analytical methods have been compared **(Nilufer and Boyacioglu 2002; Seitz 1975; Trucksess *et al.,* 1990; Whitaker *et al.,* 1996)**. However, cultural and analytical methods have not been compared in detail **(Abbas *et al.,* 2004)**. Cultural screening methods are cheap alternative techniques for detecting aflatoxigenic *Aspergillus* strains **(Abbas *et al.,* 2004)**.

The colony reverse of aflatoxin producing strains of *A. flavus* and *A. parasiticus* turned pink to red when their cultures were in contact with ammonia vapour. This colour change occurred immediately after *Aspergillus* colony were exposed to ammonia vapour **(Saito and Machida, 1999)**. No colour change was observed for non aflatoxin-producing strain of *A. oryzae*. Versicolorin, a precursor of AFB1 produced by *A. flavus* and *A. parasiticus,* is the compound that produces purple-red colour in alkaline conditions **(Saito and Machida, 1999; Hatsuda *et al.,* 1955; Lee *et al.,* 1976)**. **Shier et al. (2005)** showed that there are some other compounds that can be responsible for this colour change. It has been identified that these compounds are anthraquinone derivatives associated with aflatoxin biosynthetic pathway in *Aspergillus* species, as the biosynthetic intermediates (norsolorinic acid, averantin, averufin, versicolorin A) for AFB1. The explanation for colour change is that anthraquinone compounds behave as pH indicator dyes which will change colour **(Afsah-Hejri *et al.,* 2013)**.

The development of a yellowish orange colour in the reverse of the colonies grown on *Aspergillus* differentiation agar was a result of the reaction of ferric ions from ferric citrate with aspergillic acid molecules **(Assante *et al.,* 1981)**. This result was taken as a positive reaction for the production of aflatoxin.

The fluorescence methods are based on UV detection of AFs produced into an agar medium **(Hara *et al.,* 1974; Lin and Dianese, 1976; Davis *et al.,* 1987; Lemke *et al.,* 1989)**. Coconut agar medium (CAM) is generally used for rapid detection of aflatoxins production by *Aspergillus* spp. **(Lin and Dianese, 1976)**.

Cultivation on CAM is a primary tool to validate aflatoxins production **(De Vogel *et al.,* 1965; Arseculeratne *et al.,* 1969; Lin and Dianese, 1976; Davis *et al.,* 1987)**. Further, a blue fluorescence surrounding aflatoxigenic colonies UV light is seen on the reverse of the plates **(Almoammar *et al.,* 2013)**. However, due to the frequent false negative results, CAM is an unreliable method for detecting the toxigenic potential of *A. flavus* and *A. parasiticus* **(Taniwaki, 1996)**. **Fente et al. (2001)** showed that YES media was comparatively inducive for aflatoxin production over Czapek′s, aflatoxin producing ability (APA) media, and different coconut agar media. In contrast, **Desai and Ghosh (2003)** reported high AFB1 production in Czapek′s agar over APA and CAM.

Thin layer chromatography (TLC) is an analytical method used to validate the aflatoxins production and quantification **(Stroka and Anklam, 2000)**. TLC is the oldest of the chromatographic methods requiring less advanced and expensive equipment than other chromatographic methods, and a little simpler **(Whitaker *et al.,* 1996)**. **Atanda et al. (2005)** reported that aflatoxigenic isolates reverse exhibit a characteristic blue or blue green fluorescence under long wave UV that was confirmed by TLC. However, some non-aflatoxigenic isolates also fluorescence under UV light **(Almoammar *et al.,* 2013)**. For example, strains of *A. flavus* and *A. oryzae* produce several compounds other than aflatoxins such as asperopterin A or B **(Kaneko and Sanada, 1969)**, flavacol and deoxy-hydroxy-aspergillic acid **(Yokotsuka *et al.,* 1967)** give a blue fluorescence under UV light.

V. Conclusion

Wheat flour is an important and significant food resource for the world population. Flour quality evaluation regarding to mould infestation and mycotoxins contamination is necessary for the production of safe food. Qualitative detection of aflatoxins and aflatoxigenic fungi by cultural and analytical methods are easy, rapid and less expensive techniques to validate a primary alarm of contamination.

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Table 1: Fungal counts of wheat flour samples (n = 30) obtained from different locations in Egypt.

Isolated Fungi	El-Minia	Assiut	Sohag	Qena	Aswan	Total	Average	%
***Alternaria* spp.**	**121**	**17**	**20**	**10**	**20**	**188**	**37.6**	**1.03**
Aspergillus	**834**	**571**	**419**	**1812**	**837**	**4473**	**894.6**	**24.43**
Aspergillus clavatus	0	0	17	0	0	17	3.4	0.09
A. flavus	481	395	346	1751	276	3249	649.8	17.75
A. nidulans	0	0	0	17	0	17	3.4	0.09
A. niger	353	174	23	44	561	1155	231	6.31
A. ochraceus	0	2	0	0	0	2	0.4	0.01
A. tamarii	0	0	33	0	0	33	6.6	0.18
Cladsporium cladosporioides	0	33	7	17	0	57	11.4	0.31
***Fusarium* spp.**	**123**	**45**	**7**	**32**	**5**	**212**	**42.4**	**1.16**
***Mucor* spp.**	**12**	**33**	**163**	**217**	**8**	**433**	**86.6**	**2.36**
***Penicillium* spp.**	**3448**	**3274**	**128**	**5073**	**844**	**12767**	**2553.4**	**69.76**
Rhizopus stolonifer	**8**	**13**	**26**	**7**	**9**	**63**	**12.6**	**0.34**
Yeasts	**2**	**0**	**19**	**35**	**0**	**56**	**11.2**	**0.31**
Unknown	**13**	**0**	**2**	**18**	**20**	**53**	**10.6**	**0.29**
Total	**4561**	**3986**	**791**	**7221**	**1743**	**18302**	**3660.4**	**100**

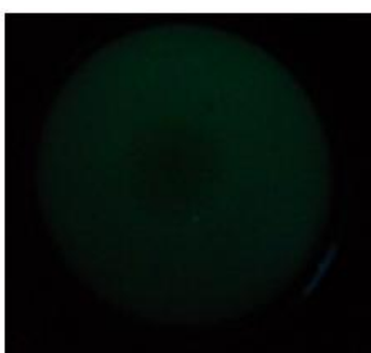

Fig. 1a: *Aspergillus flavus* on CMA, -ve fluorescence

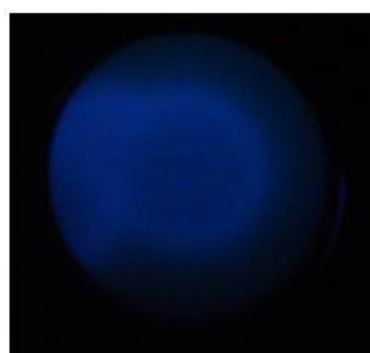

Fig. 1b: *Aspergillus flavus* on CMA, +ve fluorescence

Fig. 2a: *Aspergillus flavus* on YES, before ammonia vapour test

Fig. 2b: *Aspergillus flavus* on YES, after ammonia vapour test

Table 2: Results of screening for AF-producing strains by coconut milk agar, ammonia vapour, ADM and TLC methods.

No. of Isolate	CMA	Amm. Vap.	ADM	TLC	*Artemia salina* (%mortality)
1	-ve	-ve	+ve	-ve	-ve
2	-ve	-ve	+ve	-ve	-ve
3	-ve	+ve	+ve	-ve	20%
4	-ve	-ve	+ve	-ve	-ve
5	-ve	-ve	+ve	-ve	-ve
6	+ve	-ve	+ve	-ve	-ve
7	-ve	-ve	+ve	-ve	-ve
8	-ve	-ve	+ve	-ve	-ve
9	-ve	-ve	+ve	-ve	-ve
10	+ve	-ve	+ve	-ve	20%
11	+ve	-ve	+ve	-ve	-ve
12	-ve	-ve	+ve	-ve	-ve
13	+ve	-ve	+ve	-ve	-ve
14	-ve	-ve	+ve	-ve	-ve
15	-ve	-ve	+ve	-ve	-ve
16	+ve	-ve	+ve	+ve	100%

17	+ve	-ve	+ve	-ve	30%
18	-ve	-ve	+ve	-ve	-ve
19	-ve	+ve	+ve	-ve	-ve
20	-ve	-ve	+ve	-ve	-ve
21	-ve	-ve	+ve	-ve	-ve
22	-ve	-ve	+ve	-ve	-ve
23	-ve	-ve	+ve	-ve	-ve
24	-ve	-ve	+ve	-ve	-ve
25	-ve	-ve	+ve	-ve	-ve
26	-ve	-ve	+ve	-ve	-ve
27	-ve	-ve	+ve	-ve	-ve
28	-ve	-ve	+ve	-ve	-ve
29	-ve	-ve	+ve	-ve	-ve
30	-ve	-ve	+ve	-ve	-ve
31	-ve	-ve	+ve	-ve	-ve
32	-ve	-ve	+ve	-ve	-ve
33	-ve	+ve	+ve	-ve	-ve
34	-ve	-ve	+ve	-ve	-ve
35	-ve	+ve	+ve	-ve	-ve
36	+ve	-ve	+ve	-ve	25%
37	-ve	-ve	+ve	-ve	-ve
38	-ve	-ve	+ve	-ve	-ve
39	-ve	+ve	+ve	-ve	-ve
40	+ve	+ve	+ve	-ve	40%
41	-ve	-ve	+ve	-ve	-ve
42	-ve	-ve	+ve	-ve	-ve
43	-ve	-ve	+ve	-ve	-ve
44	-ve	-ve	+ve	-ve	-ve
45	-ve	+ve	+ve	-ve	-ve
46	-ve	-ve	+ve	-ve	-ve
47	-ve	-ve	+ve	-ve	-ve
48	-ve	-ve	+ve	-ve	-ve
49	-ve	-ve	+ve	-ve	-ve
50	-ve	-ve	+ve	-ve	-ve
51	-ve	-ve	+ve	-ve	-ve
52	-ve	-ve	+ve	-ve	-ve
53	-ve	-ve	+ve	-ve	-ve
54	-ve	-ve	+ve	-ve	-ve
55	-ve	-ve	+ve	-ve	-ve
56	-ve	-ve	+ve	-ve	-ve
57	-ve	-ve	+ve	-ve	-ve
58	+ve	-ve	+ve	+ve	**%100**
59	-ve	-ve	+ve	-ve	-ve
60	-ve	-ve	+ve	-ve	-ve
61	-ve	-ve	+ve	-ve	-ve
62	-ve	-ve	+ve	-ve	-ve
63	-ve	-ve	+ve	-ve	-ve
64	-ve	+ve	+ve	-ve	20%
65	-ve	-ve	+ve	-ve	-ve
66	-ve	-ve	+ve	-ve	-ve
67	-ve	-ve	+ve	-ve	-ve
68	-ve	-ve	+ve	-ve	-ve