Assiut Vet. Med. J. Vol. 45 No. 90, July 2001

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MYCOFLORA AND MYCOTOXINS RECOVERED FROM SALTED FISH " MOLOHA" IN UPPER- EGYPT.

(With 2 Tables)

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(Received at 25/6/2001)

الفطريات والسموم الفطرية المعزولة من الأسماك المملحة (الملوحة) في مصر العليا

محمد سيد يوسف ، رفعت محمود فرغلي

حيث أن التلوث الغذائي بالفطريات وسمومها يعتبر من أهم المشكلات الصحية لذلك أجريت هذه الدراسة الميدانية لفحص عدد ٦٠ عينة من الأسماك المملحة (الملوحة) التي يتم تصنيعها محليا في محافظات الوجه القبلي (سوهاج ،قنا ،أسوان) ميكولوجيا باستخدام وسطين غذائيين مختلفين (أحدهما شبك دكز أجار والآخر نفس الوسط مضافا إليه ١٥% كلوريدصوديوم) ،وقد وجد اختلاف واضح في عدد وأنواع الفطريات التي تم عزلها على هذين الوسطين حيث كان العدد الكلي للفطريات ٩×١٠^{٤} ، $٢,٦\times١٠^{٤}$/اجم من العينات بالترتيب ،كما تم عزل عدد ٥٦ نوعا من الفطريات بالإضافة إلي صنف واحد وكلها تنتمي إلى ١٢ جنس فطري أهمها الأسبرجلس البنسيليوم ، ايروتيم ، الميوكر. بينما تم عزل الخمائر بنسبة ١٨,٢% ، ٣% من العدد الكلي للفطريات المعزولة على الوسطين المستخدمين على التوالي ، وقد دلت نتائج الاختبار البيولوجي للسموم الفطرية على أن عدد ١٠ عينات من ٦٠ عينة (١٦,٧%) التي تم فحصها بها بقايا سموم فطرية قاتلة ليرقات الجمبري ، بينما نتائج التحليل الكروماتوجرافي للعينات أوضحت تواجد بقايا الأفلاتوكسين ب١ في عدد ٤ عينات (٤٥٠-٩٨٠ ميكروجرام/كجم) ، ب١ + ب٢ في عدد ٢ عينة (٣٤٠-٧٨٠ ميكروجرام/كجم) ، ب١ + ب٢ + ج١ + ج٢ في عينة واحدة (٤٨٠-٧٥٠ ميكروجرام/كجم) ، بينما وجدت بقايا الأسترجماتوسيستين في عدد ٤ عينات (٢٢٠-٦٥٠ ميكروجرام/كجم) ، السترنين في عينة واحدة فقط (٥٢٥ ميكروجرام/كجم) ،وقد تم مناقشة المخاطر الصحية لتواجد هذه الفطريات وسمومها في الملوحة والإجراءات اللازمة لمنعها أو التقليل من خطورتها على صحة المستهلكين.

SUMMARY

"Moloha" is a salt-fermented fish that constitute a part of popular diet in Egypt. It is a perishable food, which is easily, attacked by

microorganisms specially fungi. Therefore, 60 samples of ready-to-eat Moloha were collected from different shops at Upper Egypt Provinces. Samples were analyzed for mycological contamination to detect their potential loads from moulds and mycotoxins using two different media (Czapek's-dextrose and 15%NaCl-Czapek agar). There is a remarkably variation of fungal infestation with diverse fungal flora in tested samples. The total viable counts of moulds on the two above-mentioned media were $9x10^4$ and 2.6×10^4 colonies / g fresh sample, respectively. 56 species and one variety belonging to 12 genera were identified using dilution-plate method. The genera of highest occurrence and their respective number of species were *Aspergillus* (*A. flavus, A. niger* and *A. fumigatus*) *Penicillium* (*P. citrinum, P. puberulum, P. aurantiogriseum* and *P. roquefortii*), *Eurotium* (*E. montevidensis, E. chevalieri* and *E. herbariorum*) and *Mucor* (*M. racemosus*). On the other hand, yeast represented 18.2 and 3.0% of the total count of fungi, respectively. The obtained samples were assayed for potential presence of mycotoxins. Ten out of 60 samples (16.7%) proved to be toxic to brine shrimp (Artemia salina L.) larvae. Chromatographic analysis revealed that aflatoxin B1 was detected in four samples with concentrations ranged from 540 to 980µg/kg of fish, while aflatoxins B1 and B2 were detected in two samples (340-780µg/kg) whereas, aflatoxins B1, B2, G1 and G2 were recorded in one sample (480-750µg/kg). On the other hand, four samples were contaminated with sterigmatocystin (220-650µg/kg) while, one sample was contaminated with citrinin (525µg/kg).

Key words: *Fungi and mycotoxins contamination, salted fish, moloha.*

INTRODUCTION

Fish and their products are considered as one of the most important foodstuffs, which are the cheapest source of protein of high biological value. They comprise many of essential amino acids, minerals, vitamins, trace elements and poly-saturated fatty acids (Sedik et al., 1989).

Moloha is a famous salt-cured fish product in Egypt, which is manufactured by mixing fish and sodium chloride (25%) and allowing the mixture to be fermented naturally for 250 days. During this period the fish is hydrolyzed by the action of fish and microbial proteases (Abd-Alla et al., 1994).

Fungal contamination of salted fish is one of the main causes of their spoilage leading to great economic losses due to condemnation of such products. Moreover, many strains of moulds isolated from different types of fishes were able to provoke mycotoxins, which have potential public health hazards (Bullerman, 1979 and Ward and Baaj, 1988).

Mould genera of Aspergillus, Penicillium, Eurotium, Alternaria, Cladosporium, Paecilomyces, Rhizopus, Scopulariopsis, Mucor and Fuzarium could be isolated from salted fish (Wheeler and Hoching, 1993; Ismail et al., 1994, Munibazi and Bullerman, 1996 and Youssef, 1998).

Mycotoxins are good defined as secondary chemical metabolites of filamentous fungi which when ingested by man or animal may cause disease affecting reproduction, health and growth performance. It is already known that about 250 different mould species can produce mycotoxins, the main of which were Aspergillus, Penicillium and Fuzarium. The most frequently encountered mycotoxins in foodstuffs are aflatoxins,ochratoxins, sterigmatocystine and citrinin (Depasquale et al., 1990 and Robb, 1993).

Aflatoxins are produced mainly by some strains of A. flavus and A. parasiticus. They comprise four major toxins (B1, B2, G1 and G2) which are named according to their position and fluorescent color on thin layer chromatography (TLC) viewed under UV- light. These aflatoxins were found toxigenic and responsible for mycotoxicosis in animal and human (CAST, 1979 and McMabon, 1994).

Two mycotoxins (ochratoxin A and B) could be produced mainly by A.ochraceus that may be occurred in fermented fish and other human foodstuffs. These toxins induce acute nephrosis and liver damage in human and animals (Volk et al., 1986 and Rousseau and Petegheml, 1989).

Stregmatocystin is one of the most important mycotoxins that produced mainly by A. versicolor, A. nidulans, A.sydovii, E.amstelodami, E.chevalieri and E.rubrum. Sterigmatocystin has carcinogenic and toxicological effects on liver of man and animals (Schroeder and Kelton, 1975, Hesseltine, 1976 and Davis, 1981).

Citrinin produced mainly by P.citrinum, P.verrucosum and P.expansum and sometimes by A.terreus and A.candidus. It has been isolated from cereals causing porcine nephropathy in temperate regions (Reddy and Berndt, 1991 and Samson et al., 1996).

Therefore, the present study was planned to evaluate the rate and extent of mould contamination in salted fish (Moloha) in Upper Egypt

governorates. As well as, the isolated mould strains were screened for mycotoxin production with referring to their public health importance.

MATERIALS and METHODS

1- Collection of samples:

Sixty random samples of ready-to eat Moloha were collected from different shops in Upper Egypt Provinces (Sohag, Qena and Aswan; 20 of each). Samples were taken aseptically in sterile polyethylene bags and transferred directly to the laboratory without delay.

2- Estimation of the mould counts:

The samples were prepared according to the technique recommended by American Public Health Association (APHA, 1985). To 10 g of fish flesh, 90 ml of sterile peptone water were added and thoroughly mixed in a sterile warning blender. The mixture represented the dilution of 10^{-1}, from which tenth-fold serial dilutions were accomplished up to 10^{-6}. From the prepared serial dilutions, 1 ml was transferred into each of the previously marked duplicate Petri-dishes (one contained acidified Chapek's dextrose agar and the another contained Czapek`s agar with 15% Na Cl for estimation of halophilic moulds). After solidification the inoculated plates were incubated at 25° Cfor 5- 7 days. During the incubation period, the plates were examined daily for the "star- shape" mould growth. The total mould count per gram was obtained by counting the isolated moulds recovered from the two media applied.

3- Identification of mould isolates:

The identification of the mould genera and species were carried out by careful observation and measurements of the macroscopic and microscopic characteristics of the mould colonies according to Raper and Fennel (1965), Samson (1979), Domsch et al. (1980), Pitt and Hocking (1985) and Samson et al. (1996).

4- Screening of samples for mycotoxin production:

The mycotoxins were extracted from collected samples by using chloromethane and anhydrous sodium sulfate according to Lopez-Diaz and Flannigan (1997). Then, tenfold serial dilutions were used in the toxicity test (brine shrimp bioassay) as described by Harwig and Scott (1997). Dry eggs (0.5 g) of brine shrimp (*Artemia salina L.*) were hatched in 750 ml of natural sea water incubated at 28° C for 30- 32 h. Aliquots (0.1ml) of sea- water containing 30- 40 larvae were then added

to triplicate wells of disposable micro- plates to which 5- 20 µl of toxin solution were put. Then tenfold serial dilutions were carried out. The plates were covered with porous plastic film and incubated at 27° C. After 16- 24 h, dead larvae were counted under stereoscopic microscope; surviving larvae were killed by adding 10 µl of chloroform. The mortality calculated as percentage of dead larvae from the total. Each experiment was carried out at least thrice. Moreover, the toxin extracts were subjected to chromatographic analysis according to the method of the Association of Official Analytical Chemists (AOAC, 1980).

RESULTS and DISCUSSION

Contamination of salted fish (Moloha) by moulds and mycotoxins affect intensively the keeping quality and nutritional value of such product (Abdel-Rahman et al. 1988). It is evident from results given in Table (1) that the fungal infestation and fungal flora in the tested samples were markedly varied when inoculated into Czapek's dextrose and 15% Na Cl- Czapek agar. The total viable count of moulds and yeast per one gram sample were 9x104 ; 1.6x104 and 2.6x104 ; 0.8x103 on the two tested media, respectively. The higher count of glycophilic fungi isolated on the normal czapek medium than halophilic fungi was attributed to the effect of salt which inhibit the growth of many species. These findings are nearly similar to those reported by Morshdy et al. (1982), Atapattu and Samarajeewa (1990) and Youssef (1998), while differ than results recorded by Ismail et al. (1994). This variation in mold counts determined in Moloha samples may be due to different levels of sanitary measures adopting during handling, manufacturing and storage.

The genera of highest occurrence and their respective number of species isolated from salted fish where Aspergillus 72.9 & 63.1 (A. flavus 37.0% & 27.8% , A. niger 10.6% & 12.7% and A. fumigatus 1.5% and 0.0%) , Penicillium 3.8% & 11.7% (P. sitrinum 1.1% & 3.3% and P.puberulun 1.0% & 0.0%), Eurotium 1.3% & 18.8% (E. montevidensis 0.0% & 4.8%, E. chevalieri 1.1% & 4.5% and E. herbariorun 0.7% & 2.5%) and Mucor 1.6% & 0.0% (M. racemosus 9.0% & 0.0%) , respectively these results agree with that detected by Abed- El Rahman et al. (1988) but in lower percentages (Penicillium 38.5% and Aspergillus 26.3%). While the Penicillium spp. were 50% of the isolated fungi from the dryed salted sardinens in Tokyo (Hitokoto et al., 1976). Also, Udagawa and Tsuruda (1975) found that A. ochraaceus, A. oryzae, A. ostianus, Penicillium cyclopuim, P. pulterilli and

Syncephalastrum racemosum were the predominant fungi isolated from dry salted fish.

Various mould strains isolated from fish and fish products were found to produce mycotoxins which have potential public health hazards including livre cancer, ergotism, alimentary toxic aleukia and endemic nephropathy (CAST, 1979 and Krough, 1992) from the obtained results in Table (2), 10 out of 60 samples proved to be toxic to brine shrimp larvae. Aflatoxin B1 was detected in four samples with concentrations ranged from 450 to 980 µg /kg of salted fish. While, aflatoxins B1 and B2 were detected in two samples (340 – 780 µg /kg) whereas, aflatoxins B1,B2, G1 and G2 were recorded in one sample (480 – 750 µg /kg). On the other hand, four samples were contaminated with sterigmatocystin (220 – 650 µg /kg) but, one sample was polluted with citrinin (525 µkg). These results agree with the findings reported by Farahat and Koburger (1975) and Parasad et al. (1987).

The common mycotoxin – producing fungi associated with aflatoxins B1, B2, G1 and G2 were A. flavus, A. parasiticus and A. oruzae. While sterigmatocystin were accompanied mainly with E. montevidensis, E. chevalieri and E. herbariorum whereas, citrinin were found associated commonly with species of P. citrinum, P. expansum and A. candidus. These findings are more or less in agreement with those reported by Hussain et al. (1993) and Munimbazi and Bullerman (1996).

From this investigation it was noted that there is a higher fungal contamination associated with mycotoxin production in salted fish (Moloha) samples collected from different shops at Upper Egypt. This contamination could be attributed to improper sanitation during catching, handling, manufacturing, storage, transportation and marketing of fish. Pollution of salted fish with moulds and mycotoxin resulted in undesirable changes and rendering it unfit for marketing and constitute a high risk to consumers (Ward and Baji, 1988). Therefore the strict hygiene measures should be conducted during preparation manufacturing and storage of Molao to minimize the risk of such contamination. Also educational programs should be organized for producers and handlers to improve the quality of fish. Finally Moloha must be currently inspected in marketing for detection of mould growth in order to safeguard the health of consumers from the hazards induced by mould contamination and their mycotoxin production.

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Table (1): Total counts (TC, calculated per g fresh sample), number of cases of isolation (NCI, out of 60samples) and occurrence remarks (OR) of fungal genera and species isolated from salted fish (moloha) on Czapek's-dextrose and 15% NaCl-Czapek's-dextrose agar media at 28°C using dilution-plate method.

Genera and species	Glycophilic fungi				Halophilic fungi			
	TC	TC %	NCI	OR	TC	TC %	NCI	OR
***Aspergillus* (total count)**	65740	72.9	60	H	1824(	63.1	60	H
A.flavus Link	33380	37.0	54	H	7472	27.8	52	H
A. niger Van Tieghem	9600	10.6	44	H	3416	12.7	37	H
A. fumigatus Fresenius	1336	1.5	33	H	-	-	-	-
A. ficuum (Reich.) Hennings	4088	4.5	30	M	3192	11.9	24	M
A. parasiticus Speare	8872	9.8	29	M	2040	7.6	20	M
A. carbonarius (Bainier) Thom	3120	3.5	14	L	520	1.9	11	L
A. awamori Nakazawa	1392	1.5	14	L	200	0.7	10	L
A. duricaulis Raper & Fennel	920	1.0	11	L	-	-	-	-
A terreus Thom	480	0.5	10	L	-	-	-	-
A. candidus Link	352	0.4	9	L	-	-	-	-
A. heteromorphus Batista & Maia	296	0.3	7	R	176	0.7	5	R
A. brunneo-uniseriatus Singh & Bakshi	480	0.5	6	R	-	-	-	-
A. pulverulentus (McAlpine) Thom	280	0.3	6	R	-	-	-	-
A. phoenicis (Cda.) Thom	272	0.3	5	R	304	1.1	9	L
A. sydowi (Bain. & Sart.) Thom & Church	200	0.2	4	R	192	0.7	7	R
A. aculeatus Iizuka	160	0.2	4	R	-	-	-	-
A. brevipes Smith*	160	0.2	3	R	-	-	-	-
A. niveus Blochwitz	160	0.2	2	R	20	0.07	1	R
A. aureolatus Munt.-Cvet. & Bata	80	0.09	2	R	-	-	-	-
A. ochraceus Wilhelm	56	0.06	1	R	348	1.3	18	M
A. sulphureus (Fres.) Thom & Church	40	0.04	1	R	-	-	-	-
A. zonatus Kwon & Fennell	16	0.02	1	R	-	-	-	-
A. oryzae (Ahlb.) Cohn	-	-	-	-	336	1.2	9	L
A. versicolor (Vuillemin) Tiraboschi	-	-	-	-	24	0.09	1	R
Penicillium	3464	3.8	40	H	3160	11.7	26	M
P. citrinum Thom	1016	1.1	28	M	876	3.3	22	M
P. puberulum Bainier	920	1.02	10	L	-	-	-	-
P. brevicompactum Dierckx	488	0.5	10	L	56	0.2	3	R
P. verruculosum Peyronel	392	0.4	9	L	24	0.09	1	R
P. purpurogenum Stoll	320	0.4	5	R	-	-	-	-
P. chrysogenum Thom	96	0.1	2	R	-	-	-	-
P. duclauxii Delacroix	80	0.09	2	R	-	-	-	-
P. expansum Link ex Gray	72	0.08	2	R	280	1.0	8	L
P. rugulosum Thom	48	0.05	1	R	-	-	-	-
P. aurantiogriseum Dierckx	32	0.04	1	R	628	2.3	10	L
P. roquefortii Thom	-	-	-	-	1256	4.7	11	L
P. waksmanii Zaleski	-	-	-	-	40	0.15	1	R

Table (1): Continued

Genera and species	Glycophilic fungi				Halophilic fungi			
	TC	TC %	NCI	OR	TC	TC %	NCI	OR
***Mucor* (total count)**	1488	1.6	23	M	-	-	-	-
M. racemosus Fresenius	832	0.9	22	M	-	-	-	-
M. circinelloides Van Tieghem	640	0.7	6	R	-	-	-	-
M. hiemalis Wehmer	16	0.02	1	R	-	-	-	-
***Scopulariopsis* (total count)**	1008	1.1	12	L	109	0.4	6	R
S. brevicaulis (Sacc.) Bainier	408	0.5	10	L	48	0.18	4	R
S. constantini Bainier*	600	0.7	6	R	61	0.23	6	R
Eurotium (total count)	1188	1.3	8	L	4512	18.8	41	H
E. montevidensis **Talice & Mackinno**	-	-	-	-	1292	4.8	32	H
E. chevalieri Mangin	48	0.05	1	R	1200	4.5	20	M
E. herbariorum Mangin *	656	0.7	8	L	660	2.5	18	N
E. repens De Bary	484	0.5	4	R	630	2.3	6	R
E. amstelodami Mangin	-	-	-	-	600	2.2	5	R
E. halophilicus Christ., Papav	-	-	-	-	60	0.2	2	R
E. rubrum Konig & Bremer	-	-	-	-	38	0.14	2	R
E. verruculosum Vuillemin*	-	-	-	-	36	0.13	1	R
Rhizopus *stolonifer (Ehrenberg) Lind*	496	0.5	8	L	-	-	-	-
Spicaria *violacea Abbott* *	120	0.1	4	R	-	-	-	-
Cladosporium *cladosporioides (Fr*	120	0.1	2	R	-	-	-	-
Vries								
Alternaria *alternata (Fr.) Keissler*	80	0.09	2	R	-	-	-	-
Gymnoascus *reesii Baranetzky* *	80	0.09	2	R	40	0.15	1	R
Apiocera *chrysosperma (Bull*	-	-	-	-	48	0.18	2	R
Fries								
Trichoderma *viride Pers. Ex S. F.*	32	0.4	1	R	-	-	-	-
Yeast	1640(	18.2	20	M	800	3.0	12	L
Total count	90216				26909			
Number of genera (12)	11				6			
Number of species (56 + 1var.)	46 + 1 var.				32			

** New records to mycological laboratories in Egypt.*

OR: Occurrence remarks:

H: High occurrence; more than 30 cases out of 60 tested.
M: Moderate occurrence; between 16-30 cases
L: Low occurrence; between 8-15 cases.
R: Rare occurrence; less than 8 cases.

Table (2): Sample number (SN), sample source, biological assay (Brine shrimp), natural occurring of mycotoxins identified and common mycotoxin-producing fungi associated with the toxic salted fish samples.

SN	Sample source	Dead larvae (%)	Mycotoxins identified (µg/ Kg)	Mycotoxin producing-fungi
2	Sohag	100	Aflatoxin B_1 (960µg/ kg)	*A. flavus* and *A. parasiticus*
5		85	Sterigmatocystin (620µg/ kg)	*A. versicolor, E. montevidensis , E. herbariorum, E. repens* and *E. amstelodami*
8		95	Aflatoxins B_1 & B_2 (780 & 640µg/ kg)	*A. flavus and A. parasiticus*
22	Qena	88	Aflatoxin B_1 (540µg/ kg), Sterigmatocystin (420µg/ kg)	*A. flavus, A. parasiticus* and *E. chevalieri, E. montevidensis , E. herbariorum, E. repens* and *E. amstelodami*
24		78	Citrinin (525µg/ kg)	*A. candidus, P. citrinum* and *P. expansum*
29		100	Aflatoxin B_1 (980µg/ kg)	*A. flavus and A. parasiticus*
36		85	Sterigmatocystin (650µg/ kg)	*E. chevalieri, E. montevidensis , E. herbariorum, E. repens* and *E. amstelodami*
46	Aswan	100	Aflatoxins B_1, B_2, G_1, G_2 (480-750µg/ kg)	*A. flavus*
52		93	Aflatoxins B_1 & B_2 (660 & 340µg/ kg)	*A. flavus and A. parasiticus*
57		90	Aflatoxin B_1 (540µg/ kg) Sterigmatocystin (220µg/ kg)	*A. flavus, A. parasiticus,* and *A. oryzae, E. chevalieri, E. montevidensis , E. herbariorum and E. repens*