Mycotic quality of locally manufactured smoked herring fish

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A B S T R A C T

A grand total of 90 random samples of smoked herring fishes (30 Un packed –30 packed – 30 fillet) were collected from different markets at Monofia governorate. The samples were subjected to mycological examination for count, isolation and identification of mould and yeast. Also Molecular identification of aflatoxinogenic *A. flavus* was carried out. The incidence of mould in the examined samples (unpacked, packed and fillet) were 100%, 83.3% and 66.7%, respectively. While the incidence of yeast in the examined samples (unpacked, packed and fillet) were 90%, 66.7% and 33.3%, respectively. The mean total mould counts /g was 1.9×10^5, 2.1×10^4, 2.5×10^3 for unpacked, packed and fillets, respectively. The mean total yeast counts/g was1.7×10^5, 2.1×10^4,1.3×10^2, respectively. The results indicated that un packed smoked herring fish were more contaminated with mould and yeast. Molecular identification of aflatoxinogenic *A. Flavus* was positive for three sets of different virulence genes (*omtA, ver-1*, and *nor-1*) which involved in aflatoxin biosynthetic pathway.

Keywords: Smoked herring, Mycotoxins, Aflatoxin biosynthesis**,** *Aspergillus flavus***.**

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1. INTRODUCTION

Fish and fish products considered as a preferable source of nutritional values and highly desirable food due to its good quality animal protein content as its exceptional richness in phosphorus and calcium and its generous supply of ß- complex vitamins (Atef et al., 2011; El-Ahl-Rasha, 2010). Traditional fish products (TFPs) which usually produced by using old preserving methods such as salting, fermenting, drying and smoking greatly varies among the countries as well as within the same country by using many different applications such as differences in percentages of salt or vinegar, additives and maturing temperatures (Sevim, 2010). Smoked herring fish is the most important processed fish products in Egypt, also known as "renga" which is commercially produced by using three main steps, the first step dry salting with NaCl, the second step is partially air drying and the final step is smoking whether by using hot or cold smoking (Atef, 2013). Fungal contamination of fish is considered as the main cause of signs of spoilage as unpalatable taste and off flavor and it may constitute a public health hazard as well as many of economic losses (Hassan et al., 2009). Fungal infection leads to food spoilage such as discoloration, off-flavors, rotting and disintegration of the food structure (Martin et al., 2005). The very important aspect involved in spoilage of food by fungi is also the formation of toxic secondary metabolites –mycotoxins. Concerning the importance and diversity of their toxic effects (carcinogenic, mutagenic, nephrotoxic, immunotoxic, neurotoxic, teratogenic and hepatotoxic). The occurrence of mycotoxinogenic moulds in foods constitutes a high risk for animal and human health (Dalie et al., 2010; Sulyok et al., 2010; Tournas et al., 2011). Many genes involved in the biosynthesis of these mycotoxins have been identified and their DNA sequences have been published. PCR methods for the detection of aflatoxigenic *Aspergilli* based on the norsolorinic acid reductase encoding gene *nor*-1, the sterigmatocystin O-methyl transferase encoding gene *omt* A, the versicolorinA dehydrogenase encoding gene *ver*-1 and the regulatory gene *afl*R have been described (Chen et al., 2002). Detection of aflatoxin producing *Aspergillus* species based on *nor-1*, *ver-1* and *omt-1* genes by PCR system were reported by Geisen (1998).

Therefore, the aim of this work was evaluation of mycotic quality of locally manufactured smoked herring using mould and yeast count and PCR for

detection of toxigenic genes characterization of *Aspergillus flavus* that could produce aflatoxin.

2. MATERIAL AND METHODS

2.1. Sample

A grand total of 90 sample of locally produced smoked herring fish were randomly sampled and Purchased from different marketing sites (30 un packed -30 packed -30 fillets) ten gram from each sample. These samples were examined mycologically according to APHA (American Public Health Association) (1992) and total mould and yeast count were detected according to Cruickshank et al. (1975) on Sabouraud's dextrose agar medium then identified macroscopically, microscopically, morphologically and physiologically according to Dvorac and Atcenasek (1969) & Atcenasek, (1969) ; Finegold and martin (Finegold and Martin, 1982)e,(1982) and Harrigan (Harrigan and McCance, 1976) & McCance, (1976).

2.2. Polymerase Chain Reaction (PCR)

For identification of virulence genes for characterization of *Aspergillus flavus* (Rodrigues (Rodrigues et al., 2007) *et al.,* 2007). Table (A) shows primer sequences used for PCR identification system

2.2.1. DNA amplification for the selected virulent genes (Davari (Davari et al., 2015) et al., 2015):

The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). A total of 35 cycles was started with heating at 94°C for 5 min, and continued by denaturation for 30 secs at 94°C, annealing for 30 secs at 67°C, elongation for 30 secs at 72°C and a final extension of 10 min at 72°C. Amplified products were electrophoresed in 1% agarose gel (Sigma –USA) stained with ethidium bromide and visualized and captured on UV trans-illuminator. A 100 bp DNA ladder was used as a marker for PCR products.

3. RESULTS

Table (1) show that the Incidence of mould in the examined samples (unpacked, packed and fillet) were 30 (100%), 25 (83.3%), 20(66.7%), respectively. Table (2) show that the Incidence of yeast in the examined samples (unpacked, packed and fillets) were 27 (90%), 20 (66.7%) ,10 (33.3%), respectively. Table (1) show that the mean total mould counts /g were 1.9×10^5, 2.1×10^4, 2.5×10^3 for un packed, packed and fillets fish samples, respectively. Table (2) show that mean total yeast counts /g were 1.6×10^5, 2.1×10^4, 1.3×10^2 respectively.

Table (3 & 4) show that 80% of examined un packed smoked herring fish samples were contaminated with *Aspergillus species*, which represent as [*A. flavus* 7 isolates (23.3%), *A. niger* 6 isolates (20%) A. fumigatus 2 isolates (6.7). A. ruber 2 isolates (6.7%), A. versicolor 1 isolate (3.3%), A. nidulans 1 isolate (3.3), A. ochraceus 2 isolates (6.7%), A. terrus 3 isolates (10%)]. 50% of the examined smoked herring fish were contaminated with *Pencillium species*. Mucor, Cladosporium, Rhizopus, Fusarium, Alternaria were isolated from the examined sample in different percentage of 20%, 13.3%, 10%, 6.7%, 6.7% respectively.

For yeast species33.3% of unpacked smoked herring fish samples were contaminated with Rhodotorula spp., Candida spp. and Saccharomyces spp. were present in the same percentage of (16.7%). Meanwhile, Torulopsis spp., Trichosporum spp., Nigrospora spp. were isolated from the examined sample in different percentage of 13.3%, 10%, 6.7%, respectively**.** 46.6% of examined packed Smoked herring fish samples were contaminated with *Aspergillus species*, which represent as [*A. flavus* 5 isolates (16.7%), *A. niger*6 isolates (20%), A. fumigatus 1 isolate (3.3%), A. terrus 2 isolates (6.7%)], While 26.6% of the examined smoked herring fish were contaminated with Pencillium *species*. Mucor, Cladosporium, Rhizopus, Fusarium, Alternaria were isolated from the examined sample in different percentage of 16.6%, 10%, 10%, 3.3%, 10% respectively. For yeast species 30% of packed smoked herring fish samples were contaminated with Rhodotorula spp., Candida spp. and Saccharomyces spp. were present in the same percentage of (13.3%). Also Torulopsis spp. and Trichosporum spp. were isolated from the examined sample in the same percentage of 10%. Also Hani (2014) isolated *Aspergillus spp., Penicillum spp.*, Cladosporium *spp*., Rhizopus spp. and Mucor spp. from packaged smoked herring fish in percentage of 44%, 4.0%, 32%, 28%, 22% respectively.

Also 56.6 % of examined fillets Smoked herring fish samples were contaminated with *Aspergillus species* which represent as [*A. flavus* 5 isolates (16.7%), A. *niger*7 isolates (23.3%), A. fumigatus 2 isolates (6.7), A. ruber 1 isolates (3.3%), A. nidulans 1 isolate (3.3), A. terrus 1 isolates (3.3%)].

Table (A) Primer sequences used for PCR identification system:

Primer	Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
nor-1 (F)	aflD	5′ ACCGCTACGCCGGCACTCTCGGCAC ′3		
nor-1 (R)		5′ GTTGGCCGCCAGCTTCGACACTCCG ′3	400	
ver-1 (F)		5′ GCCGCAGGCCGCGGAGAAAGTGGT ′3		
ver-1 (R)	aflM	5′ GGGGATATACTCCCGCGACACAGCC ′3	537	(Criseo et al., 2001)
omtA (F)		5′ GTGGACGGACCTAGTCCGACATCAC ′3		
omtA (R)	aflP	5′ GTCGGCGCCACGCACTGGGTTGGGG ′3	797	

Table (1): Incidence of mould species isolated from the examined smoked herring fish samples (n=30).

Samples	No. of $^{+}$ve Samples	% of $^{+}$ve Samples	Min	Max.	Mean	S.D.
Un packed	30	100	1.9×10^{3}	9.8×10^{5}	1.9×10^{5}	2.8×10^{5}
Packed	25	83.3	2.7×10^{2}	8.2×10^{4}	2.1×10^{4}	2.6×10^{4}
Fillets	20	66.7	4.2×10^{3}	9.5×10^{3}	2.5×10^{3}	3.6×10^{3}

N.B: Percentage were calculated according to total no. of samples n=30

Table (2): Incidence of yeast species isolated from the examined smoked herring fish samples (n=30).

Samples	No. of $^{+}$ve Samples	% of $^{+}$ve Samples	Min.	Max.	Mean	S.D.
Un packed	27	90	1.3×10^{3}	8.4×10^{5}	1.6×10^{5}	2.5×10^{5}
Packed	20	66.7	2.6×10^{2}	9.2×10^{4}	2.1×10^{4}	3.1×10^{4}
Fillets	10	33.3	3.8×10	5.6×10^{2}	1.3×10^{2}	1.6×10^{2}

Table (3): classification of moulds species isolated from the examined Smoked fish samples and their incidence (unpacked, packed, fillet) (n=30).

Mould species	Un packed		packed		Fillets	
	No. of +ve Samples	%	No. of +ve Samples	%	No. of +ve Samples	%
Aspergillus spp.:	28	80%	14	46.6%	17	66.6%
A. flavus	7	23.3%	5	16.7%	5	16.7%
A. niger	6	20%	6	20%	7	23.3%
A. ochraceus	2	6.7%	0	0%	0	0%
A. fumigates	2	6.7%	1	3.3%	2	6.7%
A .ruber	2	6.7%	0	0%	1	3.3%
A.terrus	3	10%	2	6.7%	1	3.3%
A.nidulanc	1	3.3%	0	0%	1	3.3%
A.versicolor	1	3.3%	0	0%	0	0%
Penicilliumspp	15	50%	8	26.6%	5	16.6%
Mucorspp	6	20%	5	16.7%	4	13.3%
Cladosporiumspp	4	13.3%	3	10%	2	6.7%
Rhizopusspp	3	10%	3	10%	1	3.3%
Fusariumspp	2	6.7%	1	3.3%	1	3.3%
Alternariaspp	2	6.7%	3	10%	1	3.3%

Table (4): classification of yeast species isolated from the examined Smoked fish samples and their incidence (unpacked, packed, fillet) (n=30).

Yeast species	Un packed		packed		Fillets	
	No. of +ve Samples	%	No. of +ve Samples	%	No. of +ve Samples	%
Rhodotrula spp.	10	33.3%	9	30%	2	6.7%
Candida spp.	5	16.7%	4	13.3%	1	3.3%
Saccharomyces spp.	5	16.7%	4	13.3%	0	0%
Torulopsis spp.	4	13.3%	3	10%	1	3.3%
Trichosporum spp.	3	10%	3	10%	2	6.7%
Nigrospora spp.	2	6.7%	0	0%	0	0%

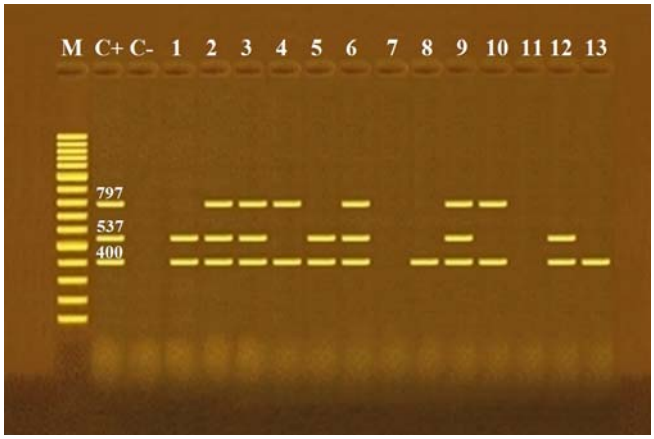

Fig. (1): Agarose gel electrophoresis of multiplex PCR of nor-1 (400 bp), ver-1 (537 bp) and omtA (797 bp) virulence genes for characterization of *Aspergillus flavus*. Lane M: 100 bp ladder as molecular size DNA marker. Lane C+: Control positive strain for nor-1, ver-1 and omtA genes. Lane C-: Control negative. Lanes 1,2,3, 5,6,9&12: Positive *A. flavus* strains for nor-1 and ver-1 genes. Lanes 2, 3, 6 & 9: Positive *A. flavus* strains for nor-1, ver-1 and omtA genes. Lane 4 & 10: Positive *A. flavus* strains for nor-1 and omtA genes. Lane 8 & 13: Positive *A. flavus* strains for nor-1 gene. Lanes 7 & 11: Negative *A. flavus* strains for nor-1, ver-1 and omtA genes.

While 16.6 % of the examined smoked herring fish were contaminated with Pencillium *species*. Mucor, Cladosporium were isolated from the examined sample in different percentage of 13.3%, 6.7% respectively. Rhizopus, Fusarium, Alternaria isolated in the same percentage 3.3%. For yeast species 6.7% of fillets smoked herring fish samples were contaminated with Rhodotorula spp., Candida spp., Torulopsis spp., Trichosporum spp., were isolated from the examined sample in different percentage of 3.3%, 3.3%, 6.7% respectively. Fig. (1) showing positive result for *omt-A, ver-1 and nor-1* coding genes for the key enzymes in aflatoxin B1 biosynthesis.

4. DISCUSSION

Herring as a food vehicle is subjected to contamination with variable genera and species of mould and yeast, therefore the preventive and strictly measures of significant concern intended to eradicate or at least to minimize this contamination or its hazard effects to protect human from consuming the ready-to-eat smoked herring from the probable danger of both acute and chronic toxic effects (Mounir et al., 2011).

Many strains of moulds were recorded to constitute a public health hazard due to mycotoxin production such as aflatoxin, ochratoxin, patulin and zearalenone (Hassan et al., 2009). Which have carcinogenic effect, the high mycotoxins levels could lead to liver cancer while subacut levels are responsible for liver diseases and organs damage (Youssef, 1998).

It is evident from data present in table (1) that the Incidence of mould in the examined samples (unpacked, packed and fillet) were 30 (100%), 25 (83.3%), 20(66.7%) respectively. While it is evident from data present in table (2) that the

Incidence of yeast in the examined samples (unpacked, packed and fillets) were 27 (90%), 20 (66.7%), 10 (33.3%) respectively. The results obtained are nearly similar to those obtained by El-Sayed (1995). Lower results were obtained by Abd El- Maksoud et al. (2010). While Edris et al. (2012) *et al.,* (2012) reported that the rate of mycotic contamination in the examined Vacuumed packed herring 52% and 68% for fillet and negative yeast. It is evident from data present in table (1) that the mean total mould counts /g were 1.9×10^5, 2.1×10^4, 2.5×10^3 for unpacked, packed and fillets fish samples respectively. Table (2) show that mean total yeast counts /g were 1.6×10^5, 2.1×10^4, 1.3×10^2 These results were nearly similar to results obtained by Adebayo-Tayo et al. (2008); El-Sayed (1995); El-Shater (1994); El-Zahaby (2007); Mounir et al. (2011)and Hani (2014), while lower results were obtained by Abd-Eldayem-Wafaa (1999); Abd El- Maksoud et al. (2010); Awad-Hoda et al. (1998); Edris (1996); Ibrahim (2000); Mahmoud (2010); Safaa (1999) *and* Nyarko et al. (2011).

Smoked fish might be easily contaminated with mould growth specially un packed smoked herring, it may be due using contaminated salt, packaging and bad handling (Plahar et al., 1991).

It is evident from data present in table (3&4) that 80% of examined un packed Smoked herring fish samples were contaminated with *Aspergillus species*, which represent as [*A. flavus* 7 isolates (23.3%), *A. niger* 6 isolates (20%) A. fumigatus 2 isolates (6.7). A. ruber 2 isolates (6.7%), A. versicolor 1 isolate (3.3%), A. nidulans 1 isolate (3.3), A. ochraceus 2 isolates (6.7%), A. terrus 3 isolates (10%)], While 50% of the examined smoked herring fish were contaminated with Pencillium *species*. Mucor, Cladosporium, Rhizopus, Fusarium, Alternaria were isolated from the examined sample in different percentage of 20% ,13.3%,10% ,6.7%, 6.7 % respectively. For yeast species 33.3% of unpacked smoked herring fish samples were contaminated with Rhodotorula spp. Candida spp. and Saccharomyces spp., were present in the same percentage of (16.7%), Meanwhile Torulopsis spp., Trichosporum spp., Nigrospora spp. were isolated from the examined sample in different percentage of 13.3%, 10%, 6.7% respectively. Such results are nearly similar to those obtained by Atef et al. (2011); El-Sayed (1995); El-Shater (1994); El -Gazzar et al. (2005); Mounir et al. (2011); Nayel (2007); Safaa (1999); Youssef and Farghaly (2003) and Radwa (2012) . While Edris (1996) (1996) who isolated *Pencillium species* and *Aspergillus species* from the examined samples in percentage of 80%, 60% respectively. Also Ibrahim (2000) isolated the same species from 70%, 50% of examined sample respectively., Salem (2004) isolated the same species from 58% ,48% of examined sample respectively. Moreover Mahmoud (2010) isolated *Aspergillus spp. and Penicillum spp.* (50%), (46.66%) from smoked fish respectively. Also Hani (2014) isolated *Aspergillus spp., Penicillum spp*., Cladosporium *spp*., Rhizopus spp. and Mucor spp. from unpackaged smoked herring fish in percentage of 48% , 42% , 38% , 32% and 22% respectively .

In the Egyptian markets, most of the smoked fish, are stored in wooden boxes at ordinary room temperature for a sufficient time, predisposing them to mould and yeast growth (El-Sayed, 1995). Also 46.6% of examined packed Smoked herring fish samples were contaminated with *Aspergillus species*, which represent as [*A. flavus* 5 isolates (16.7%), *A. niger* 6 isolates (20%), A. fumigatus 1 isolate (3.3%), A. terrus 2 isolates (6.7%)] ,While 26.6% of the examined smoked herring fish were contaminated with *Pencillium species*. Mucor, Cladosporium, Rhizopus, Fusarium, Alternaria were isolated from the examined sample in different percentage of 16.6%, 10%, 10%, 3.3%, 10% respectively. For yeast species 30% of packed smoked herring fish samples were contaminated with Rhodotorula spp., Candida spp. and Saccharomyces spp. in the same percentage of 13.3%. Also Torulopsis spp. and Trichosporum spp. were isolated from the examined sample in the same percentage of 10%. Also Hani (2014) isolated *Aspergillus spp., Penicillum spp.*, Cladosporium *spp*., Rhizopus spp. and Mucor spp. from packaged smoked herring fish in percentage of 44% , 4.% , 32% , 28% , 22% respectively .

Also 56.6 % of examined fillets Smoked herring fish samples were contaminated with *Aspergillus species*, which represent as [*A. flavus* 5 isolates (16.7%), A. *niger*7 isolates (23.3%), A. fumigatus 2 isolates (6.7), A.ruber 1 isolates (3.3%) , A. nidulans 1 isolate (3.3) , A. terrus 1 isolates (3.3%)] ,While 16.6 % of the examined smoked herring fish were contaminated with *Pencillium species*. Mucor, Cladosporium were isolated from the examined sample in different percentage of 13.3%, 6.7% respectively. Rhizopus, Fusarium, Alternaria isolated in the same percentage 3.3% . For yeast species 6.7% of fillets smoked herring fish samples were contaminated with Rhodotorula spp., Candida spp, Torulopsis spp., Trichosporum spp., were isolated from the examined sample in different percentage of 3.3%, 3.3%, 6.7% respectively. These results came in disagreement with those reported by Edris *et al.,* (2012) who can't isolate yeast from herring fillets in jar.

Fish treated by a bad hygienic measures may be prone to contamination with microorganisms such

as bacteria and fungi and presence of *Aspergillus flavus* in the samples might probably make its consumption hazardous to health (Abd El-Maksoud et al., 2010). The presence of species of Aspergillus could be attributed to the prevalence of their spores in the atmosphere (Adebayo-Tayo et al., 2009).

Unhygienic handling such as the use of old news prints, cement papers and polyethylene bags are all sources of contamination of fish, which constitute a public health hazard (Edris et al., 2012; El-Zahaby, 2007). It was evident from Fig. (1) that specific primers *for A. flavus* were designed based on the *omt-A, ver-1 and nor-1* genes coding for the key enzymes in aflatoxin B1 biosynthesis and used to detect the fungus. Most of samples are Positive *A. flavus* strains for nor-1, ver-1 and omtA genes. These results agree with those reported by Ebaid (2014).

The expression of aflatoxin genes has been applied for detection and differentiation of aflatoxigenic strains. so, various regulatory and structural aflatoxin pathway genes in *Aspergillus parasiticus* and *Aspergillus flavus* have been targeted (Degola et al., 2007; Scherm et al., 2005; Sweeney et al., 2000). Aflatoxins are secondary metabolites, which are produced by *Aspergillus flavus*, when taken by human. It could induce hepatitis and hepatic enlargement. Also are known to be carcinogenic and mutagenic (Abbas et al., 2004).

In conclusion, the obtained results in the present study indicated that unpacked smoked herring fish were more contaminated with mold and yeast. Moreover, PCR is a sensitive technique for detection of virulence genes enter in mycotoxins biosynthetic pathway. Also the present study demonstrated that unhygienic handling and improper sanitation during catching, handling, processing, storage, transportation, distribution and marketing of fish. All sources of contamination of fish constitute a public health hazard.

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