Official Journal Issued by Faculty of Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: https://bvmj.journals.ekb.eg/

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Original Paper

Mycological quality of processed fishes in Kafrelshiekh governorate with special reference to Aspergillus flavus virulent factors

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ARTICLE INFO

Keywords

Aflatoxin,

Aspergillus flavus,

Canning, Packing,

PCR,

Processed fish products

Received 20/11/2024

Accepted 06/01/2025

Available On-Line 01/04/2025

ABSTRACT

This study aimed to determine the prevalence of *Aspergillus flavus* in some processed fishes with reference to its virulence factors genetically. For this purpose, sixty random samples of processed canned fish products (tuna and sardine), salted fish products (salted mugil spp.; feseikh and sardine), and smoked fish products (packed and non-packed renga)—"10 of each"—were obtained from different markets in Kafrelshiekh Governorate. Collected samples were exposed to mycological examination to determine the incidence of *A. flavus* according to the standard method, followed by molecular identification of aflatoxigenic isolates. *A. flavus was found in 20% of the feseikh, 10% of the salted non-canned sardine, 10% of the unpacked renga, 20% of the canned tuna, and 10% of the canned sardine. It was not found in the packed renga.* Molecular identification of aflatoxigenic *A. flavus* isolates based on their content of *omtA, nor-1,* and *ver-1* genes revealed that the *omtA* and *nor-1* genes were positively detected in all of the examined isolates (100%); whereas, the *ver-1* gene was detected in only 42.86% of the examined isolates. These results concluded that packed renga has a lower fungal contamination without isolation of *A. flavus,* and subsequently it is safer than unpacked renga, feseikh, unpacked salted sardine, canned tuna, and canned sardine. Moreover, the use of high-quality raw materials, the application of high-standard hygienic conditions during processing, and proper storage conditions are essential for safer processed fish products.

1. INTRODUCTION

Fish and fish products constitute a major source of high-quality health-needed nutrients (Pal *et al.*, 2018; Allam *et al.*, 2019). For their nutritive value, there has been a global rise in the need for fish and fish products during the last decade (Elhafez *et al.*, 2020).

Some old ways of making processed fish, like salting, drying, and smoking, are very different from one place to another because of the amounts of salt, vinegar, and other ingredients used to preserve the fish and the temperatures used during the processing (Fitri et al., 2022). In Egypt, among the most preferred processed fishes are canned tuna, salted feseikh and sardine, and smoked renga, which are also consumed in several countries, and it is relatively safe even in the developed countries (Hamad *et al.*, 2023).

Feseikh is a traditional Egyptian salted fish. Sodium chloride used in salted fish can enhance flavor, as it can modulate the activity of enzymes associated with organoleptic parameters (Wang *et al.*, 2021). Renga (smoked herring fish) manufacture includes dry salting with sodium chloride, partial air drying, and hot or cold smoking (Osheba, 2013).

High moisture and nutrient content in addition to higher pH are among the main factors making fish more susceptible to fungal spoilage, which may be loaded from contaminated fish feeds prepared from ingredients that are occupied by toxigenic fungi such as *Aspergillus flavus* (*A. flavus)* and *A. parasiticus* (Udomkun *et al.*, 2017, Ali *et al.*, 2022). Moreover, the hygienic quality of the used raw ingredients, handling, and storage are essential factors in the hygienic quality of fish products.

The most important signs of food spoilage induced by fungal contamination are unpleasant taste and flavor, discoloration, rotting, and, in some cases, it could cause a public health hazard for animals and humans through the production of mycotoxins (Abdelhady *et al.*, 2017). These toxins have the potential to induce toxicity in several organs/tissues, such as the kidney, liver, and nerves, and in some cases, they have tumorigenic and teratogenic properties (Mavrommatis *et al.*, 2010).

Aflatoxins produced by *Aspergillus species* are the most dangerous mycotoxins, which are commonly found in foods poorly stored in higher temperatures and humidity; that consumption of food containing aflatoxins could cause liver damage and subsequently cancer (Wu and Mitchell, 2016).

Several genes that participated in aflatoxin synthesis in aflatoxigenic Aspergillus species were detected by PCR, revealing it as a useful diagnostic technique for the aflatoxigenic Aspergilli. These genes include the norsolorinic acid reductase-encoding gene (*nor-1*), the sterigmatocystin O-methyl transferase-encoding gene (*omt A*), and the versicolorin A dehydrogenase-encoding gene (*ver-1*) (Ibrahim *et al.*, 2017).

Continuous monitoring of fungal contamination in fish products, especially those that produce aflatoxins, is an imperative necessity for consumer safety. Therefore, the present study was conducted to determine the incidence of *A. flavus* contaminating some common processed fish products, with special reference to the aflatoxin-inducing genes (*omtA*, *ver-1*, and *nor-1*).

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2. MATERIAL AND METHODS

Statement of Ethics

2.1. Samples

A total number of sixty random samples of processed canned fish products (tuna and sardine), salted fish products (feseikh and sardine), smoked fish products (packed and non-packed renga) "10 of each" were purchased from different markets in Kafrelshiekh Governorate.

2.2. Detection of A. flavus

2.2.1. *The mycotic examination of the processed fish samples*

It was performed as described by ISO 21527-1 (2008). Total fungi count was done using Dichloran Rose Bengal agar with chloramphenicol (DRBC: OXOID) then were incubated at upright position at 25^{O}C for 5 -7 days. *A. flavus* isolates were identified morphologically, biochemically, and microscopically as previously described by Pitt and Hocking (2009); who described *A. flavus* colony to be 65-70 mm in diameter, floccose powdery or granular in texture, greenish yellow surface color; as well as containing globose to ellipsoid conidia with radiating head cleistotheca microscopically.

2.2.2. Molecular identification of aflatoxin predisposing genes in Aspergillus flavus isolates

It is worth noted that *A. flavus* isolates used in the PCR examination were sorted as the following: 1st and 2nd isolates were obtained from feseikh; 3rd isolate was from salted sardine; 4th isolate was from canned sardine; 5th isolate was from unpacked renga; 6th and 7th isolates were from canned tuna.

PCR was used to amplify *A. flavus* virulence genes (*omtA*, *ver-1*, and *nor-1*). DNA was isolated from fungi samples using QIAamp DNeasy Plant Mini kit (Catalogue no.69104) following manufacturer protocol. The primers were designed based on previous publication (Norlia *et al.*, 2019) as presented in Table 1.

The prevalence of *A. flavus* was recorded as a ratio to the number of each examined processed fish samples (10), and to the total number of the examined samples (60) as the following equation:

$$\frac{\text{No. of examined samples} - \text{No. or positive samples}}{\text{No. of examined samples)} \times 100}$$

3. RESULTS

Results, as was recorded in Table (2), revealed isolation of *A. flavus* from 20%, 10%, 10%, 20% and 10% of the examined feseikh, salted non-canned sardine, unpacked renga, canned tuna, and canned sardine, respectively; while, it was not detected in packed renga.

Table 1. Sequences of primers used for amplifying virulence genes of *A. flavus*

Gene	Sequence (3'---5')	Amplified product (bp)	Reference
omtA	GGCCCGGTTCCTTGGCTCCTAAGC	1024	Norlia *et al.*, 2019
	CGCCCCAGTGAGACCCTTCCTCG		
nor1	ACCGCTACGCCGGCACTCTCGGCAC	400	
	GTTGGCCGCCAGCTTCGACACTCCG		
ver1	GCCGCAGGCCGCGGAGAAAGTGGT	537	
	GGGGATATACTCCCGCGACACAGCC		

Table 2. Incidence of *A. flavus* isolated from the examined processed fishes (n = 10).

Processed fishes	Positive samples	
	No.	%
Canned sardine	1	10*
Canned tuna	2	20*
Feseikh	2	20*
Packed renga	ND	--
Salted sardine	1	10*
Unpacked renga	1	10*
Total	7	11.7**

*- Prevalence of A. flavus in relation to number of each examined fish product (10).
**- Prevalence of A. flavus in relation to total number of the examined samples (60).

According to the Egyptian standards for canned tuna (E.S 804: 2016), canned sardine (E.S 287: 2017), feseikh (E.S 1725-1: 2005), salted sardine (E.S 1725-2: 2005), and smoked renga (E.S 288: 2020), processed fish products should be free from fungal growth and its mycotoxins. Based on this, Fig. (1) showed the rate of acceptability of the examined samples, where all of the examined packed renga was fit for human consumption; whereas, 80%, 90%, 90%, 80% and 90% of the examined feseikh, salted non-canned sardine, unpacked renga, canned tuna, and canned sardine were fit for human consumption, respectively.

According to the findings of the molecular detection of the *omtA*, *ver-1*, and *nor-1* genes, all isolates of *A. flavus* had the presence of the *omtA* and *nor-1* genes, with a prevalence of 7/7, 100%. However, the predominance of the ver-1 gene was only found in three isolates of *A. flavus* (3/7, 42.86%). One sample of canned sardine (1/1, 100%), salted sardine (1/1, 100%), and Feseikh (1/2, 50%) all contained ver-1 (Figs. 2 , 3 & 4).

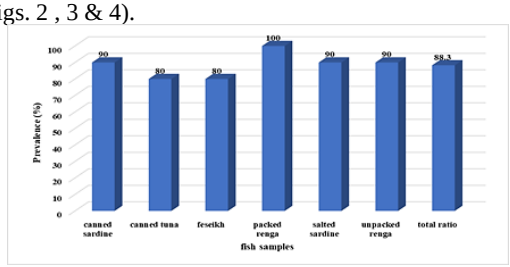

Fig. (1). Acceptability ratio of the examined processed fish products according to the Egyptian standards

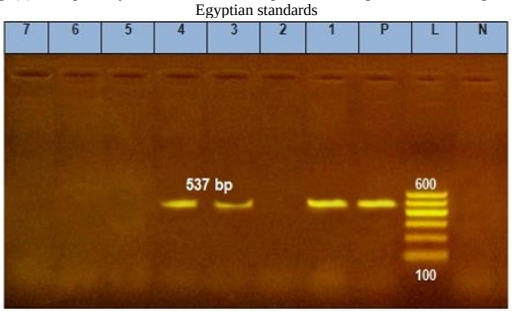

Fig.2. Agarose gel (1.5%) electrophoresis displays 1024 bp PCR products of the *omtA* gene. Lanes 1–7: examined isolates of *A. flavus* (all positive for the *omtA* gene); Lane N: negative control (deionized water); Lane L: 100 bp ladder as a molecular size standard; and Lane P: positive control (*A. flavus*).

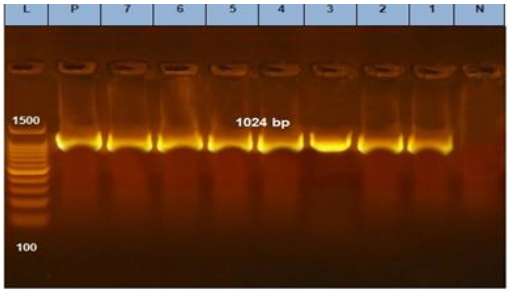

Fig.3. Agarose gel (1.5%) electrophoresis displays 537 bp *ver-1* gene PCR products. Lanes 1–4: positive *A. flavus* isolates for the *ver-1* gene; Lanes 2–7: negative *A. flavus* isolates for the *ver-1* gene; Lane N: negative control (deionized water); Lane L: 100 bp ladder as a molecular size standard; and Lane P: positive control (*A. flavus*).

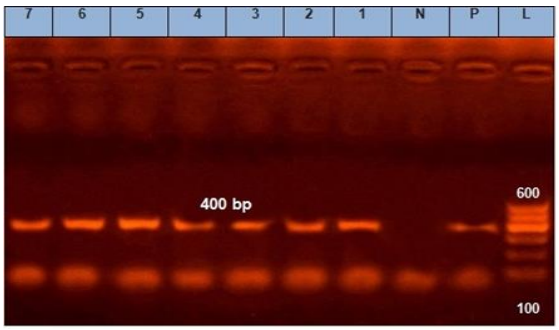

Fig.4. Agarose gel (1.5%) electrophoresis displays 400 bp PCR products of the *nor-1* gene. Lanes 1–7: positive isolates of *A. flavus* for the *nor-1* gene; Lane N: negative control (deionized water); Lane L: 100 bp ladder as a molecular size standard; and Lane P: positive control (*A. flavus*).

4. DISCUSSION

Fish and fish products contain high moisture contents, making them more susceptible to fungal contamination, which could result in mycotoxicosis (Marijani *et al.*, 2019). Among the processed fishes in Egypt, those prepared by salting, drying, and smoking, such as feseikh and smoked herring, are more prone to fungal contamination (Edris *et al.*, 2012). These processed fish could be easily contaminated with fungi during the addition of contaminated salt, packaging, and unhygienic handling, which could cause a public health hazard, especially when processed fish is contaminated with *A. flavus* (Edris *et al.*, 2017; Ibrahim *et al.*, 2017).

Referring to the present results, the incidence of *A. flavus* contamination indicated that packed renga is less contaminated with fungi and could be safer for human consumption as it is free from the aflatoxigenic *A. flavus*. Consistent with these findings, Ibrahim *et al.* (2017) documented a higher prevalence of *A. flavus* in the unpacked renga (23.3%) than in the packed renga (16.7%). However, this previous study also detected a few *A. flavus* isolates in the packed renga. Similar higher contamination with A. *flavus* was reported in unpacked renga by Atef *et al.* (2011) and Mounir *et al.* (2011); whereas, Kusmarwati *et al.* (2021) found that *Aspergillus spp*. was detected in salted fish by the prevalence of 36.8%, and Rady *et al.* (2024) detected *A. flavus* in 35.1% of the examined renga samples. The higher fungal contamination of unpacked renga could be attributed to the storage method, which used wooden boxes at normal room temperature for a long time. In the same line, the higher prevalence of *A. flavus* in feseikh samples may be referred to its unhygienic storing and frequent exposure to the ambient temperature and surrounding contaminants (Ibrahim *et al.*, 2017). The high frequency of mold suggests that inadequate sanitary practices were used during handling, processing, and preparation (Shaltout *et al.*, 2022).

Meat and meat products naturally contain secondary fungal compounds called mycotoxins. Toxigenic molds, such as Aspergillus species, are responsible for producing aflatoxins and ochratoxins, which are harmful to both human and animal health due to their potential to cause mutagenesis, hepatotoxicity, nephrotoxicity, cancer, and teratogenicity (Agriopoulou *et al.*, 2020). In addition, people with significantly compromised immune systems, several mold species pose a serious risk of respiratory infections (Kraft *et al*., 2021).

About twenty-seven enzyme processes are involved in the polyketide pathway that produces aflatoxins. It has been shown that *nor-1*, *ver-1*, and *omtA* are implicated in the gene sets that regulate these processes. The norsolorinic acid ketoreductase needed to convert Versicolorin A's (10-hydroxyl) group from the 10-keto group of Norsolorinic Acid (*nor*) (*verA*) is encoded by the gene *aflD* (*nor-1*). *VERA* is converted to Sterigmatocystin (ST) by aflM (*ver-1*), which is predicted to encode a ketoreductase; O-methyltransferase, which is one of the primary genes in charge of converting ST into O-methylsterigmatocystin (OMST), the precursor for the production of aflatoxin, is encoded by aflP (*omtA*) (Zhou *et al*., 2024).

To detect aflatoxigenic isolates in the processed fishes, a PCR assay was conducted to screen for the presence of virulence genes (*omtA*, *ver-1*, and *nor-1*) participating in aflatoxin biosynthesis. Interestingly, *omtA* and *nor-1* virulence genes were present in all *A. flavus* isolates with a prevalence of (7/7, 100%). However, the *ver-1* gene was only detected in three *A. flavus* isolates with a prevalence of (3/7, 42.86%) represented by isolates of feseikh, salted sardine and canned sardine. Consistent with our results, Ibrahim *et al.* (2017) also detected *the omtA*, *ver-1*, and *nor-1* genes in all *A. flavus* isolates from renga with higher prevalence in unpacked renga, and Thasniaty *et al.* (2024) recorded positive detection of aflatoxigenic *A. flavus* genetically in their examined salted fish samples in Indonesia.

5. CONCLUSIONS

The present study revealed a lower fungal contamination of packed renga than unpacked renga, feseikh, unpacked salted sardine, canned tuna, and canned sardine. Unlike other processed fishes, packed renga showed no growth of *A. flavus*, and thus its consumption could be safer. However, the other processed fishes, especially the unpacked renga, had higher contamination with fungi, including the most dangerous and highly virulent *A. flavus* strains. Thus, these processed fish could constitute a public health hazard for humans, and consumers should be aware of the dangers of pathogens in these processed fish. Therefore, strict hygienic measures should be applied during their handling, processing, storage, transportation, and marketing. Moreover, Good Manufacturing Practices (GMPs) should be followed to guarantee the safety and quality of processed fish. Molecular characterization of aflatoxigenic *A. flavus* proved to be effective in identification of toxigenic fungal contaminants of fish processing chain.

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