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Molecular Characterization of Pathogenic Mould and Yeast Isolated From Poultry Farms with Detection of Mycotoxins Residues

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In the present study, a total of 50 samples of poultry rations and drinking water in front of the birds (25 for each) were collected from various private broiler farms at Giza governorate. The collected samples were subjected to isolation and identification of contaminant fungi, screening of poultry feeds for aflatoxin B_1 residues, assessment of isolated *A. flavus* strains for aflatoxin production as well as molecular detection of the *aflR* gene in isolated *A. flavus* strains by polymerase chain reaction and molecular detection of the pathogenic *Candida lusitaniae*. The results showed 100 rations samples were highly contaminated with moulds, but were free from yeasts. The prevalence of mould and yeast from drinking water samples were 48% and 88%, respectively. The total mould count (log 10 CFU/g) obtained from poultry feed samples collected from poultry houses was higher than that of drinking water samples. Total mould count (log 10 CFU/g) in poultry feed samples ranged from 1.8 to 4.4 (log 10 cfu/g) while in drinking water samples ranged from 1 to 1.8 (log 10 cfu/ml). On the other side, it was found that the total yeast count of the examined drinking water ranged from 1.8 and 4. 2 (log 10 cfu/ml). Altogether, one hundred and three isolates representing 9 genera were recovered during this study. The most frequent fungi were from the genus Aspergillus, were *A. flavus* (17.9 %) appeared to be the most prevalent. The predominant candida species isolated from drinking water samples were *C .lusitaniae* (21.6 %), *C. tropicals* (16..2 %), *C. pelliculosa* (13.5 %), *C .famata* (10.8 %) and *C. krusei* (8.2%). Where Torulopsis spp. and Rhodotorula spp were also isolated with a prevalence of 4 (10.8%) and 7 (18.9 %).The aflatoxin B_1 residues were detected in 7 (28%) rations samples with a mean value of 43 µg/ kg. Gamma radiation cause complete inhibited germination of *A. flavus* spores at 9 kGy.

Introduction

Poultry production has shown a tremendous growth all over the world due to its protein value, easily digestible, more palatable and the cheapest price, especially in developing countries [1].

Aspergillus flavus is one of the most important pathogenic fungi that contaminate grains and poultry feed mixtures which cause serious problems such as mycotoxicosis due to aflatoxins production [2]. Aflatoxins are one of the most prevalent and dangerous mycotoxins defined as, secondary metabolites produced by the aflatoxigenic fungi such as *A. flavus, A.parasiticus, A. nomius, A. bombycis* and *A. pseudotamarii* [3]. Although 18 different aflatoxins have been identified, only aflatoxins B_1, B_2, $G_1$and G_2 are significant contaminants of agricultural commodities and pose a potential risk to poultry [4]. Among these, aflatoxin B_1 is of the greatest concern due to its carcinogenic, mutagenic, teratogenic, and immunosuppressive effects that increases susceptibility of toxicated birds to

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bacterial, viral and parasitic infections. Moreover AFB_1 has hepatotoxic, nephrotoxic effects [5] and growth inhibitory effects [6]. Poultry are the most sensitive of all farm animals to the toxic effects of even small amounts of aflatoxin B_1 [7].

Candidiasis, otherwise known as thrush, is a fungal disease caused by yeasts of the genus Candida [8]. Among them, six are most frequently isolated. While *C. albicans* is the most abundant and significant species, *C. tropicalis, C. glabrata, C. parapsilosis, C. krusei and C. lusitaniae* have also been implicated as causative agents [9]**.** Transmission of Candida mainly occurs via faecal contamination of feed and water.

The polymerase chain reaction (PCR) facilitates in vitro amplification of the target sequence. The main advantages of PCR is that organisms need not to be cultured, at least not for long period prior to their detection, target DNA can be detected even in a complex mixture, no radioactive probes are required, it is rapid, sensitive and highly versatile

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aflatoxin biosynthesis. The PCR reaction was targeted aflatoxin synthesis regulatory gene (aflR1) since it is conserved in *A. flavus* and *A. parasiticus*, indicating the possibility of detection of both species with the same PCR system (primers/reaction) [11]**.**

Different methods of preservation such as fumigation, fungicide application and heat treatment have been used to eliminate mould contamination, but none of them offered complete control of pathogenic and toxigenic fungal species. Meanwhile, it has been proved by several authors that gamma irradiation is a promising method applied for the decontamination and preservation of plant crops and different food and feedstuffs considering the disadvantages caused by alternative methods [12 &13]. Up to date, many measures have been taken in order to decrease fungal contamination and mycotoxin production in poultry feed, including radiation methods. Radiation is a physical treatment depends on exposure of packed or bulk food to a source of ionizing radiation to effectively reduce microbial contamination [14]. The treatment efficacy depends on several factors; including poultry feed composition, irradiation dose, and the number and type of microorganisms. Radiation methods can inactivate microorganisms that decompose food, including bacteria, filamentous fungi and yeasts.

The present study was planned aiming for molecular characterization of pathogenic mould and yeast isolated from feed and water of poultry farms, and gamma irradiation was evaluated for its in vitro antifungal activity against *Aspergillus flavus* on crushed corn meal to extend the shelf-life of rations.

Materials and Methods

Collection of samples

A total of 50 samples of poultry ration and drinking water in front of the birds were collected from various private broiler farms at Giza governorate. The rations were separately collected in sterile polyethylene bags, while water samples in sterilized bottles. The collected samples were subjected to the following examinations:

(1) Determination of the total mould and yeast count according to [15].
(2) Isolation and Identification of isolated moulds and yeasts: according to Pitt and Hocking, 2009 [16] and Cruickshank et al., 1975 [17] and Koneman et al., 1992 [18] respectively.
(3) Detection of aflatoxin B_1 residues in poultry ration according to Roberts and Patterson, 1975 [19] and Howell and Taylor [20].
(4) Screening of the isolated A.flavus strains: for production of aflatoxin in yeast extract sucrose medium (YES) according to [21].
(5) Polymerase Chain Reaction (PCR) amplification

Four isolates of candida species and *A. flavus* were examined by polymerase chain reaction (PCR). The amplified fragments were purified using Gene Jet PCR purification kit; Ferments (CAT NO. KO701). Published strains in GenBank were selected as Reference sequences. Sequencing was performed by Macrogen Company (South Korea) and identification of homologies between nucleotide and amino acid sequences, our stains were compared with other strains published on GenBank using BLAST 2.0 and PSI-BLAST search programs, (National Center for Biotechnology Information NCBI" http://www.ncbi.nlm.nih.gov/), respectively the obtained nucleotide sequences comparisons and

their multiple alignments with reference strains as well as the deduction of amino acid sequences were done using the BioEdit sequence alignment editor [22]. Clustal X software for multiple sequence alignment [23]. Clustal W software for multiple sequence alignment [24].ClustalV [25] and MegAlign (DNASTAR,Lasergene®,Version 7.1.0,USA [26]. The phylogenetic trees were constructed using MegAlign for tree reconstruction of sequences by Neighbor-joining method based on Clustal W. Bootstrapping values were calculated using a random seeding value of 111. Clustal V was used when end gaps were faced. Sequence divergence and identity precent were calculated by MegAlign the structural character of our protein sequence was identified by protean (Dnaster, Lasergene®, Version 7.1.0 USA).

(6) Gamma radiation sensitivity determination

Gamma radiation was assayed for fungicidal activity against the fungal strain A. flavus isolated from poultry ration in Mycology Department at Animal Health Research Institute, Giza. Confirmation of Aspergillus genera was done by subculture onto Malt Extract Agar and Czapek yeast agar. Furthermore, confirmation of *A.flavus* was carried out by Single Spore method using three cultures media: Malt Extract Agar (M.E.A) at 25°C, Glycerol Nitrate Agar (G25N) at 25^0C and Czapek yeast Agar (CYA) at 5°C and 37°C. Using the identification keys of **Pitt and Hoching** [27] observation has been made after 5-7 days. Spores from 7 day old *A. flavus* cultures grown at 25^0C on slants of Sabouraud dextrose agar were harvested in sterile 0.1 % Tween 80, then spores were counted in a haemocytometer and the final concentration of the suspension was adjusted to 1×10^6 spores/ml. Homogenous fungal suspension of *A. flavus* (1×10^6 spores/ml) were inoculated in crushed corn meal with *A. flavus*. Several trails were applied to detect the optimum dose of gamma radiation required for complete elimination of the *A. flavus*. The inoculated crush corn meal with *A. flavus* were exposed to gamma rays emitted from cobalt -60 source at room temperature at a dose rate of 5, 7 and 9 kGy/hr. the irradiation process was carried out using the facilities of the National Centre for Radiation Research and Technology Egypt. After treatment with Gamma radiation at different doses ??? missing words??? from each was cultivation from crushed corn meal and control (unirradiated crouched corn meal) to determine the optimal elimination dose.?? Incomplete phrase??

Results and Discussion

Moulds and mycotoxins contamination of feed and feed ingredients occurs worldwide due to the ubiquitous nature of these microorganisms, they cannot be totally eliminated. The presence of moulds and mycotoxins in poultry feeds results mainly from the use of raw materials that may be contaminated during the pre-harvest and/ or post-harvest periods [28].

The results recorded in figure (1) showed that all the samples of rations are highly contaminated with moulds. Their prevalence reaches up to 100% in ration, but all ration samples free from yeasts. While the prevalence of mould and yeast from drinking water samples were 48% and 88%, respectively.

From the results present in table (2), it was noticed that total mould count (log 10 CFU/g) obtained from poultry feed samples collected from poultry houses was higher than that of drinking water samples. Total mould count (log 10 CFU/g) in poultry feed samples ranged from 1.8 to 4.4 (log 10 cfu/g) while in drinking water samples, it was ranged from 1 to 1.8 (log 10 cfu/ml).On the other side , it is evident that the total yeast count of the examined drinking water ranged from 1.8 and 4. 2 (log 10 cfu/ml), But yeast failed to be detected in examined poultry feed.

Table (1): Primers designed for candida and Aspergillus

Primer pairs	Oligonucleotide sequence (5′→3′)	product size (bp)
Forward ITS1 Reverse ITS2	- TCCGTAGGTGAACCTGCGG1 3′′5 -TCCTCCGCTTTATTGATATG 3′′5	~550bp
Forward *aflR1* Reverse *aflR1*	5´-AACCGCATCCACAATCTCAT- 3´ 5´- AGTGCAGTTCGCTCAGAACA 3´	798 bp

Table (2): Total moulds and yeast count (log 10 CFU/g or /ml) of examined samples (N=25)

Type of sample	No. of examined samples	Mould count (log CFU/g)			Yeast count (log CFU/g)		
		% of +ve samples	Min.	Max.	% of +ve samples	Min.	Max.
Ration samples	25	100	1.8	4.4	0	0	0
Drinking water	25	48	1	1.8	88	1.8	4.2

In the present study, results recorded in figure (1) and table (2) showed that most of the samples of poultry ration were contaminated by moulds. These results agree with Gayakwad *et al.*2001 [29] who detected the presence of fungi in 94% of the examined samples, while Saleemi *et al*. 2010 [30] reported that incidence of moulds in feed was 69.66 and 73.10 % from commercially prepared and farm mixed feeds respectively. Similar results concerning the fungal counts in poultry feed have been reported by Labuda and Tančinová (2006) [31] and Vesna *et al*., (2010) [32] who mentioned that the total fungal count was 4.3 log cfu /g in the most of investigated poultry feed samples.

Altogether, 103 isolates belonging to 25 fungal species representing 9 genera were recovered during this study. Table (3) revealed that the prevalence of mould genera isolated from poultry feed and water samples encompassed 9 genera Aspergillus spp., Cladosporium spp., Fusarium spp., Mucor spp., Penicillium spp., Neosartorya fischeri, Rhizopus spp., Scopulariopsis spp. and Trichoderma species. The most frequent fungi from the genus Aspergillus, *(A. flavus*, 17.9 %). Along with results from the similar studies reported by Labuda and Tancinova (2006); Oliveira *et al.* (2006) [31& 33]and Saleemi *et al*. (2010) [30] who determined that Aspergillus species were the most predominant mould species isolated from commercially farm and mixed feeds followed by Penicillium, Fusarium and Alternaria sp. While the most isolated species by Vesna *et al*. (2010) [32] were Fusarium (56.09%) and Aspergillus (54.35%), followed by Rhizopus (40%), Penicillium (30.87%), Mucor (30.04%) and the least frequently isolated species were Alternaria spp. (3.48%).

As shown in table (4), it was obvious that the predominant candida species isolated from drinking water samples were *C .lusitaniae (21.6 %), C. tropicals (16..2 %), C. pelliculosa (13.5 %), C .famata (10.8 %)* and *C. krusei (8.2%).* Where Torulopsis spp. and Rhodotorula spp were isolated from drinking water samples with prevalence of 4 (10.8%) and 7 (18.9 %).

Candidiasis otherwise known as thrush, is a fungal disease caused by yeasts of the genus Candida having nearly 200 species [8]. Among them, six are most frequently isolated, while *C. albicans* is the most abundant and significant species. At the same time *C. tropicalis, C. glabrata, C. parapsilosis, C. krusei and C. lusitaniae* have also been implicated as causative agents. Transmission of Candida mainly occurs via faecal contaminated feed and drinking water causing candidiasis. Candida spp. may become part of the inhabitant flora of the mouth, oesophagus and crop [34& 35].

The residues of aflatoxin B_1 in examined poultry rations samples were determined and recorded in figure (2). The aflatoxin B_1 residues could be detected in7 (28%) poultry rations samples as well as the minimum detected level of aflatoxin B_1 was10 µg/ kg and the maximum was 100 µg/ kg with a mean value of 43 µg/ kg (table 5).

Probably the two most important environmental factors enhancing mould growth and mycotoxin production are hot and humid conditions (24^{o}C and 28^{o}C and at least 17.5 %.) These conditions approximate the ambient climatic conditions in most parts of Africa [36].

Table (3): Prevalence of individual members of mould in examined samples

Isolated moulds species	Ration samples		Drinking water	
	No. of + ve isolates	% of + ve isolates	No. + ve isolates	% of + ve isolates
Aspergillus species:				
A. clavatus	2	2.4	4	21.1
A. flavus	15	17.9	0	0
A. fumigates	3	3.6	0	0
A. ochraceus	2	2.4	0	0
A. niger	8	9.5	3	15.8
A. sydowii	2	2.1	0	0
Cladosporium spp.	5	5.2	2	10.5
Fusarium species:				
F. chlamydosporium	2	2.4	0	0
F. moniforium	2	2.4	0	0
F. oxysporum	2	2.4	0	0
F. poae	2	2.4	0	0
F. solani	3	3.6	0	0
Mucor spp.	8	9.5	2	10.5
Neosartorya fischeri	2	2.4	0	0
Penicillium species				
P. chrysogenum	3	3.6	0	0
P. citreonigenum	3	3.6	0	0
P. citrinum	3	3.6	2	10.5
P.corylophilum	5	6.0	3	15.8
P. oxalicum	4	4.7	0	0
P. paxilli	4	4.7	0	0
P. purpurogenum	2	2.4	2	10.5
P. simplicissimum	3	3.6	1	5.3
Rhizopus spp.	3	3.6	0	0
Scopulariopsis spp.	4	4.7	0	0
Trichoderma spp.	2	2.4	0	0
Total	84	100	19	100

N.B: percentages were calculated in relation to the total number of examined samples.

Table (4): Prevalence of individual members of yeast in examined samples

Isolated yeast species	Ration samples		Drinking water	
	No.	% of +ve samples	No.	% of +ve samples
C .famata	0	0	4	10.8
C. krusei	0	0	3	8.2
C .lusitaniae	0	0	8	21.6
C. pelliculosa	0	0	5	13.5
C. tropical	0	0	6	16..2
Torulopsis spp.	0	0	4	10.8
Rhodotorula spp	0	0	7	18.9
Total	0	0	37	100

N.B: percentages were calculated in relation to the total number of examined samples.

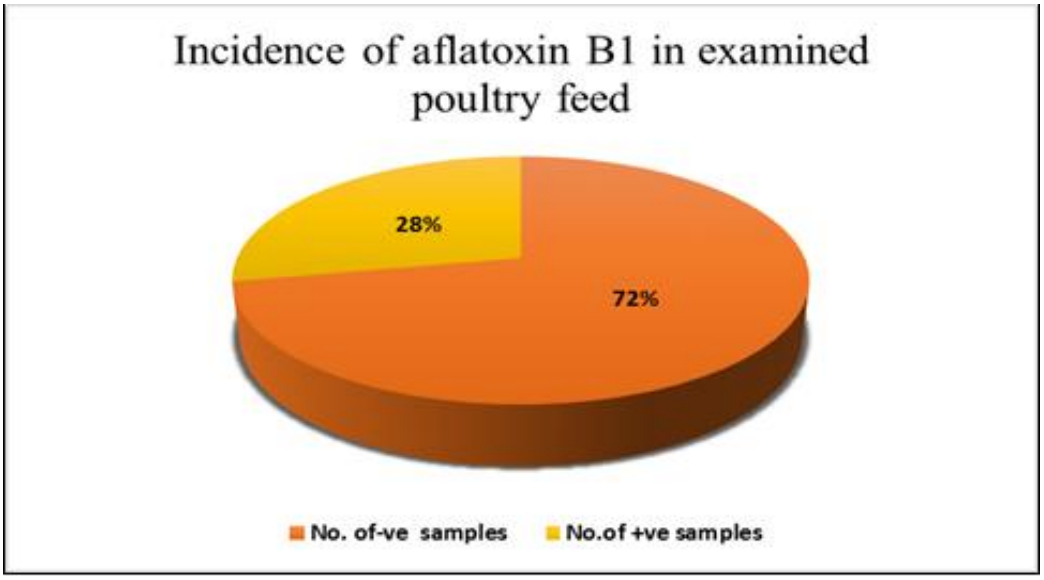

Fig (2): Incidence of Aflatoxin B_1 residues in examined poultry feed

Table (5): Aflatoxin B_1 residues in examined poultry rations (µg/ kg)

Samples	No. of examined samples	*Positive AFB_1*		*Min.*	*Max.*	*Mean*	*± SE.*
		No.	%				
Poultry ration	**25**	**7**	**28**	**10**	**100**	**43**	**± 28.6**

It was found that the major hosts of *A. flavus* among food and feed commodities are cereal grains, peanut, cotton seed and protein sources such as , cotton seed meal, soya been meal, sunflower meal, corn gluten meal and palm kernel meal [37]. Akande *et al*., (2006) mentioned that aflatoxin producing fungi utilize the nutrients present in the ingredients for their metabolism and propagation, and thereby reduce the nutritional quality of the feed ingredients [38].

In the present study, we examined 3 strains of *A.flavus* by using *aflR* gene primer. The *aflR* gene PCR products were found in 2 of these three *A. flavus* strains were successfully amplified and sequenced one strain only. These PCR results suggested that the *aflR* gene is absent or significantly different in some *A.flavus* strains figures (3& 4). In this study, figures (5&6) describes 99.1 % nucleotides sequence similarity for the one sequenced strain AFla-MRM-EG-3 and greater than 94.8 % similarity for the entire *aflR* gene sequence of the sequenced strain in comparison to 18 strains of Aspergillus spp. These findings are consistent with the results obtained by [10]. ITS-region sequences were obtained for Candida isolate (*C. lusitaniae*). Identification of the *C. lusitaniae* by GenBank showed 100% concordance with ITS15.8S-ITS2 rDNA sequencing, and molecular method showed 98% concordance to biochemical methods and 100 % similarity for sequence analysis.

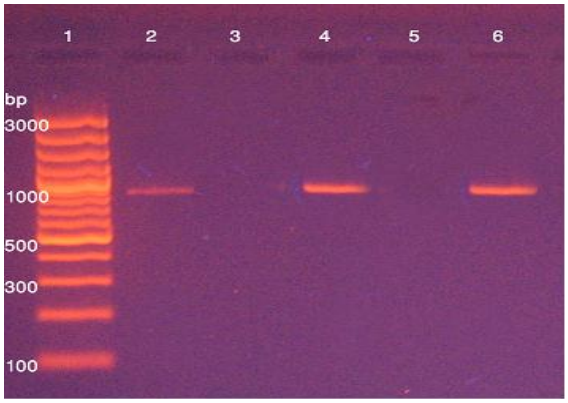

Fig (3): Agarose gel electrophoresis of PCR amplification products showing 798 bp of aflR1 gene with primer specific to the aflatoxin regulatory gene. Lane (1): 100 bp DNA ladder ,Lane (2): Control positive ,Lane (3): Control negative ,Lane (4 & 6): strain of A. flavus positive for aflR1 gene and Lane (5) strain A. flavus negative for aflR1 gene

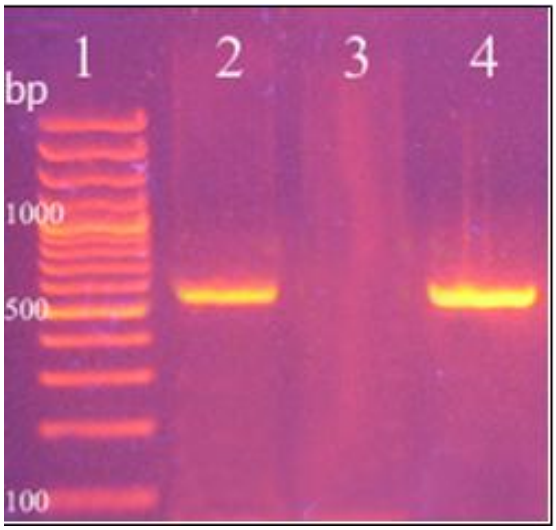

Fig. (4): Agarose gel electrophoresis of Candida sp. DNA of PCR amplification products showing 550 bp , Lane 1: 100bp DNA ladder. Lane 2: Control Positive,Lane3: Control Negative and Lane 4: sample (C. lusitaniae)

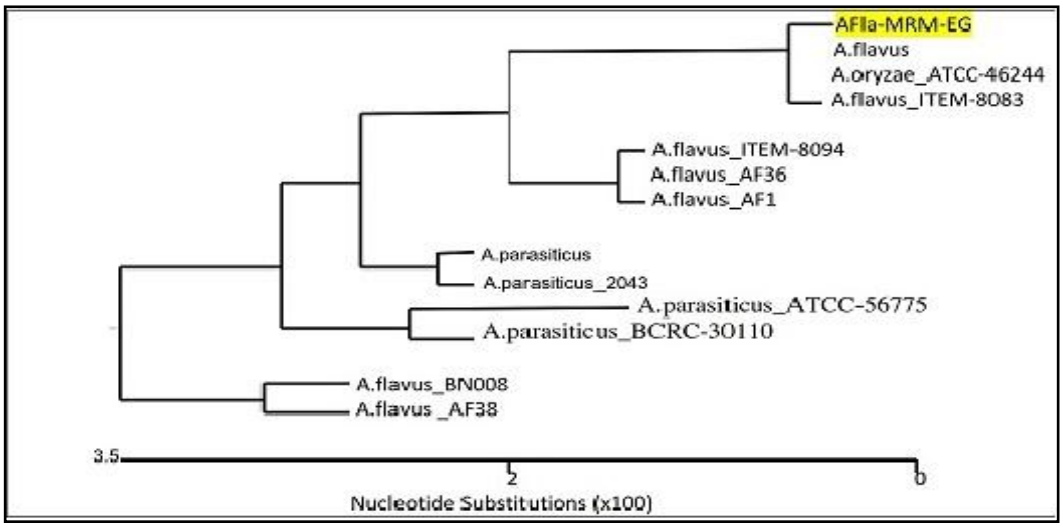

Fig. (5): Phylogenetic tree of nucleotide sequences of A. flavus

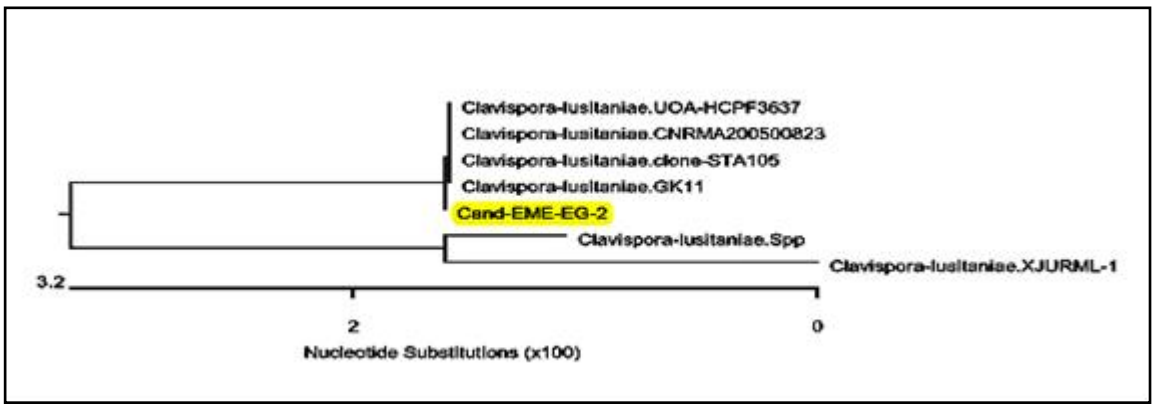

Fig. (6): Phylogenetic tree of nucleotide sequences of C. lusitaniae

Table (6): Effect of gamma irradiation on A. flavus inoculated crushed corn meal

Doses of rays by (kGy/h)	Inoculated crushed corn meal
0	1×10^6
5	5.3×10^3
7	1×10
9	ND

ND = Not detected

Gamma irradiation has been known as a successful method for food preservation though lowering or completely elimination of microorganisms which may cause food spoilage and deterioration *A. flavus* is known to be a common aflatoxigenic fungi. Growth of this fungus and subsequent aflatoxin production represents a serious public health concern to the human beings. Table (6) showed that prior to radiation, the colony formation was found to be 1×10^6 cfu/g, where as it was decreased to 5.3×10^3, 1×10 cfu/g and germination of *A. flavus* spores complete inhibited at 9 kGy of gamma radiation. Mahmoud et al (2016) mentioned that radiation of millet grains at a dose level higher than 0.5 kGy caused significant ($p < 0.05$) reduction on the percentage of fungal

incidence [39]. Furthermore, it was reported that doses of 1.5 and 3.5 kGy reduced the number of fungi in many raw fruits and vegetables [40]. Hilmy *et al.* (1995) concluded that radiation process peanuts with doses up to 1 kGy inhibit the incidence of mycelium and toxins secretion [41]. Salem and Shalaby (2016) mention that gamma irradiation dose of 2.5 KGy was very effective and greatly reduced both mycelial dry weight and aflatoxin production, while 3.5 KGy completely inhibt the growth of *A.flavus and* subsequently aflatoxin production [42]. Reduction in the fungal incidence rate in crushed corn meal after radiation might be due to high sensitivity of the *A.flavus* and mould to gamma radiation, since the radiation process causes direct and indirect damage to the DNA [43].

Conclusions

Poultry are very sensitive to aflatoxin and small quantities of aflatoxin in feed can cause increased mortality, reduction in body weight gain, feed intake, egg production and egg weight. *A.flavus* is one of the most important causes of aflatoxin production in poultry feed. Conventional methods using Aspergillus differential Medium (ADM) however could differentiate aflatoxin producers and non-producers, but this method is time consuming and sometimes it may fail to identify the aflatoxin production because of instability of aflatoxin producing strains growing on culture media. PCR is a molecular technique to differentiate aflatoxin producing strains from non-aflatoxin producing strains of ***A. flavus, A. parasiticus*** and other Aspergilli. In conclusion, by using amplification and sequencing of the *aflR* gene, we have demonstrated that the *aflR* gene cannot be amplified from some strains. Candidiasis, an alimentary tract disease usually occurs as a secondary infection, is seen worldwide with usually low morbidity and mortality rates. However it affects the poultry in terms of high production losses. Prevention of disease depends on adherence to several good managemental practices. So that, the gamma radiation process cause complete inhibited germination of *A. flavus* spores at 9 kGy.

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