**Determination of aflatoxins by HPLC and the identification of biosynthetic *nor-1 gene* of aflatoxinsin poultry products by PCR assay**

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**Abstract:**

Aflatoxins residues in different poultry products were determined using HPLC technique and the expression of biosynthetic nor-1 gene of these aflatoxins by PCR assay. 90 different chicken products were analyzed for presence of aflatoxins (AFB1, AFB2, AFG1 and AFG2) using highly accurate, robust, selective, sensitive and precise HPLC-FLD assay. Incidence of aflatoxins in examinedsamplesshowed that the level of AFB1, AFB2 and AFG2 were 10 % for each and of AFG1 was16.7%. The highest aflatoxins levels of analyzed samples were found in liver. Expression of *nor-1 gene* of aflatoxins in polluted poultry products by PCR assay.A Real time PCR (RT-PCR) technique was applied to detect the gene expression of structural nor-1, which catalyzed the first step in the biosynthetic pathway of aflatoxins. The obtained results showed a very strong relationship between the presences of aflatoxins biosynthetic genes as analyzed by multiplex PCR and aflatoxins determination by HPLC/FLD.

**Key words:**Aflatoxins- HPLC- *nor1 gene*- PCR

**Introduction:**

Mycotoxins are secondary metabolites produced by fungi such as *A.flavus, A.parasiticus, A.ochraceous, F.tritinctum, F.poae* and *penicilliumspp*.;aflatoxins, ochratoxins, patulin and T-2 are the most mycotoxins typescausing problems and associated with calamity outbreaks through the field (**Hassan *et al*., 2015)**.

Aflatoxins attracted special attention by their ability to contaminate hundred kinds of poultry and poultry products such as chicken liver, nuggets, wings and thigh. These toxins production ability had been reported in different spp. of genus *Aspergillus*fundamentally in section *Flavigp*. (**Rodrigues *et al.,* 2009**).

Aflatoxins are produced by various spp. of *Aspergillus*, particularly *A.parasiticus* and *A.flavus* (**Blesa *et al.,* 2003; Kalcher *et al.,* 2007 and Yu *et al.,* 2008**), about 60% of isolates were aflatoxins producer (**Razzaghi-Abyaneh *et al.,* 2006**). The major aflatoxins are called aflatoxin B1, aflatoxin B2, aflatoxin G1 and aflatoxin G2 (AFB1, AFB2, AFG1 and AFG2) and their two metabolites AFM1 and AFM2, but AFB1 is the most toxigenic one. AFB1 and AFB2are produced by *A*.*flavus*, while *A*.*parasiticus* produce the four aflatoxins (**Dorner, 2004**).

Aflatoxins are extremely toxic, highly mutagenic and carcinogenic compound, chemically. They are a group of di-fura-roco-marine derivative. The cause why gene expression of aflatoxins producer and non-producer strains is obvious to be fundamental to follow the inculpated strains and its way in the environment. The most established assay for estimation of aflatoxins amounts in food and feed, is the HPLC technique depending on chemical and physical properties of mycotoxins provides anaccurate, precise and specificmethod to determine the aflatoxinsconcentrations in polluted food and feed. Many researches explain biological, analytical and genetic methods (**Hassan *et al*., 2015**).

Ochratoxin has four types (A, B, C and D) have been widely detected in food products. Ochratoxin A (OTA) is the most dangerous one. OTA is produced by *A. spp., P.verrucosum* and *P.nordicum* (**Frisvad and Samson 2000; Larsen *et al.*2001; Abarca *et al*., 2001 and Castella *et al.,* 2002**). Ochratoxin is immunosuppressive, teratogenic and carcinogenic effects; it is considering fertility inhibitor and mutagenic as it can pass placenta (**IARC, 1993 and WHO, 2002**). OTA has been considered as the major cause responsible for the “Balkan Endemic Nephropath” in humans (**Kuiper-Goodman & Scott, 1989**).

The development of sensitive, accurateand rapid method for identification of incriminated aflatoxigenic species in foods and feed is main aim to avoid any potential health hazard and control mycotoxins (**Valasek and Repa, 2005**). Polymerase chain reaction (PCR) and real time Polymerase chain reaction (RT-PCR) have proved to be highly precise and rapid bimoleculartechniques for genes expression or their transcripts participate in biosynthesis of mycotoxins by *A.flavus* and *A.parasiticus*(**Fente *et al*. 2001; Somashekar *et al*., 2004** and **Scherm *et al.,* 2005**)**.**

The aim of the present study was to quantity Aflatoxins in 90 different poultry products by HPLC assay and to detect structural *nor-1* gene expression by RT-PCR in contaminated samples*. Nor-1* gene is a key structural gene in biosynthetic pathway of aflatoxins it encodes an enzyme that catalyze the conversion of the first stable aflatoxin biosynthesis intermediate norsolorininc acid to averantin.

**Material and methods:**

**Materials:**

***Samples:***

A grand total of 90 different chicken products (chicken wings, chicken nuggets, chicken liver and chicken thigh) "15 of each". The samples were collected from different supermarkets, Elsharqia. The samples were placed in plastic bags then transferredin an ice boxto the lab. without delay.

The collected samples were analyzed for the presence of aflatoxins (AFB1, AFB2, AFG1 and AFG2)

***Material used for extraction of DNA***

**QIAamp DNeasy Plant Mini kit Catalogue no.69104**: DNeasy Plant Kits provide a fast and easy way to purify DNA from plant and fungal tissue. Up to 100 mg of tissue can be processed using the DNeasy Plant Mini Kit.

**Ethanol 96%:**Applichem

***PCR Master Mix used for conventional PCR***

**Emerald Amp GT PCR mastermix (Takara)** Code No. **RR310A** Contains: **(**Emerald Amp GT PCR mastermix (2x premix) and PCR grade water).

***Oligonucleotide primers used in conventional PCR***

They have specific sequence and amplify a specific product as shown in Table (1).

Table (1): Oligonucleotide primers sequences Source:Metabion (Germany).

|  |  |  |  |
| --- | --- | --- | --- |
| **Gene** | **Sequence** | **Amplified product** | **Reference** |
| aflR | AAC CGC ATC CAC AAT CTC AT | 800 bp | **Bintvihok, *et al*., 2016** |
| AGT GCA GTT CGC TCA GAA CA |

***DNA Molecular weight marker***

**Gene ruler 100 bp DNA ladder** (cat. no. SM0243) supplied from Fermentas**.**

Number of bands: 10

Size range: 100-1000 bp.

**Material used for agarose gel electrophoresis**

**Agarose 1.5% (Sambrook *et al.,* 1989):** A multi-purpose, high gel strength agarose suitable for a wide range of molecular biology techniques. As it has high gel strength and exclusion limits, multi ABgarose could effectively separate large DNA fragments with reduced running times. This in turn means less band diffusion, a problem often associated with long running times. It was prepared as follow: (1.5 g Agarose powder (ABgene) and 100ml TBE.

**Ethedium bromide solution 10 mg / ml (Sambrook *et al*., 1989):** 10 mg Ethedium bromide powder (Sigma) then add 1.0 ml sterile DDW, mix and stored covered at 4˚C**.** It was added to melted agarose to reach a final concentration of 0.1-0.5 μg/ml.

**Tris borate EDTA (TBE) electrophoresis buffer (1x ) (WHO, 2002):** 10.78 gmofTris buffer, Fluka), 5.5 gm Boric acid, Fluka) and 0.82 gm EDTAdiNA, Winlab).It was completed up to 1 liter with DDW, pH was checked up. If the pH was out of the range of 8-8.6, a new solution was prepared again. Any change in ion concentration would affect the DNA migration through the gel.

**Methods:**

**Quantification of aflatoxins by HPLC:**

**Preparation of samples for residue study:**

At the time of assay, partially thaw frozen tissues at room temperature (23oc) for 30 minutes and blend in a food processor for 20-30 seconds at high speed to obtain a uniform paste-like consistency.

**Extraction and clean-up:** (**Brera *et al*., 2011)**

**1)** Weight 5gm into a polypropylene tube.

**2)** Add 0.5 g of sodium chloride and 25 mL of methanol (80%).

**3)** Mix at high speed for 3 min. Filter the extract through filter paper.

**4)** Pipette 3 mL of filtrate and dilute with 3 mL of PBS. Mix thoroughly.

**5)** Centrifuge the diluted sample for 10 min at 10,000 rpm.

**6)** Apply 4 mL of the diluted sample to the conditioned immunoaffinity column (IAC) and wash with 1 mL of PBS. Elute mycotoxins in a 2-step procedure. First, apply 1.0 mL methanol to the IAC and let it flow through under gravity. Collect eluate in calibrated 5 mL volumetric flask. Wait 1 min and apply a second portion of 1.0 mL methanol. Pass air through the column to collect the remaining few drops.

**7)** Fill the 5 mL volumetric flask to the mark with DW, mix well, and store the sample at +4◦C prior to analysis.

**Validation of analytical method:**

The method was validated according to **ICH, 2005** guidelines

*Limit of Detection (LOD) and Quantification (LOQ):* They were calculated from Based on standard deviation of intercept (S) and slope (b) LOD = 3.3×S/b and LOQ = 10×S/b

*System Suitability Test:* Relative standard deviations of the retention time, tailing factor, number of theoretical plates, peak area, and capacity factor were measured to test system suitability

*Accuracy and recovery:* The standard additions at different concentrations are prepared by adding known quantities of AFs & OCA. Those samples are analyzed against standard solutions of same concentrations. The accuracy is then calculated from the test results as a percentage recovery.

*Precision:* It is determined using 5 replicates of each standard solutions and evaluating the relative standard deviation of repeatability (RSD %).

*Selectivity and specificity:* Verification of selectivity is conducted by evaluating the standard addition on blank matrix. Acceptance criteria: there is no interference between the pure standard and peaks of any impurities or extracted

solvents.

 **Irradiation effects on mycotoxins reduction: (Aziz *et al*., 2004)**

Chicken product samples of high positive results were packed into polyethylene pouches and irradiated, in three replications, with doses of 6, 8 and

10 KGy by using 60CO gamma rays (Gamma Cell mold 220 apparatus, NCRRT,

Nasr City, Cairo, Egypt)

 **Expression of *nor-1 gene* of aflatoxins and *ocrA* gene of ochratoxin in polluted poultry products by a real time PCR (RT-PCR) technique:**

**Extraction ofDNA** (QIAamp DNeasy Plant Mini kitinstructions):

1. Freeze 100 mg of the fungal tissue at –80°C/ 24 hrs for later processing.
2. Add fungal material and a tungsten carbide bead to a 2 ml safe-lock tube. 400 μl Buffer AP1 and 4 μl RNase A stock solution (100 mg/ml) were added. Tubes were placed into the adaptor sets, which are fixed into the clamps of the TissueLyser. Disruption was performed in two 1–2 min. at high-speed (20–30 Hz) shaking steps.
3. The mixture was incubated for at 65°C/ 10 min and mixed 2 or 3 times during incubation byinverting tube.
4. Add 130 μl Buffer P3 to the lysate, mixed, and incubated for 5 min on ice.
5. The lysate was centrifuged for 5 min at 14,000 rpm.
6. The lysate was pipetted into the QIAshredder Mini spin column (lilac) placed in a 2 ml collection tube, and centrifuged for 2 min at 14,000 rpm.
7. The flow-through fraction from step 16 was transferred into a new tube without disturbing the cell-debris pellet.
8. Add 1.5 volumes of Buffer AW1 to the cleared lysate, and mixed by pipetting.
9. Pipette 650 μl of the mixture from step 8 (including any precipitate that was formed) into the DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged for 1 min 8000 rpm and the flow-through was discarded.
10. Step 9 was repeated with the remaining sample. The flow-through and collection tube were discarded.
11. The DNeasy Mini spin column was placed into a new 2 ml collection tube.
12. Add 500 μl Buffer AW2 was added to the DNeasy Mini spin column, and centrifuged for 2 min at 14,000 rpm to dry the membrane.
13. The DNeasy Mini spin column was transferred to a 1.5 ml or 2 ml microcentrifuge tube, and 50 μl Buffer AE were directly pipette onto the DNeasy membrane. It was incubated for 5 min at room temperature (15–25°C), and then centrifuged for 1 min at 8000 rpm to elute.
14. Step 13 was repeated once.

**Preparation of conventional PCR MasterMix:**

(According to Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit as shown in table (2)

Table (2): components of PCR mastermix

|  |  |
| --- | --- |
| **Component** | **Volume/reaction** |
| Emerald Amp GT PCR mastermix (2x premix) | 12.5 *μl* |
| PCR grade water | 4.5 *μl* |
| Forward primer (20 pmol) | 1 *μl* |
| Reverse primer (20 pmol) | 1 *μl* |
| Template DNA | 6 *μl* |

**Cycling conditions of the primers during cPCR:**

Temperature and time conditions of the two primers during PCR are shown in Table (3).

Table (3): Cycling conditions of the different primersduring conventional PCR

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Primary denaturation** | **Secondary denaturation** | **Annealing** | **Extension** | **No. of cycles** | **Final extension** |
| 95˚C  5 min. | 94˚C  30 sec. | 50˚C  1.25 min. | 72˚C  1.40 min. | 35 | 72˚C  10 min. |

**DNA Molecular weight marker**

The ladder was mixed gently by pipetting up and down. 6 μl of the required ladder were directly loaded.

**Agarose gel electrophoreses (Sambrook *et al*., 1989) with little modification**

Electrophoresis grade agarose (1.5 g) was prepared in 100 ml TBE buffer in a sterile flask, it was heated in microwave to dissolve all granules with agitation, and allowed to cool at 70˚C, then 0.5μg/ml ethedium bromide was added and mixed thoroughly.

The warm agarose was poured directly in gel casting apparatus with desired comb in apposition and left at room temperature for polymerization.

The comb was then removed, and the electrophoresis tank was filled with TBE buffer. Twenty μl of each PCR product samples, negative control and positive control were loaded to the gel. The power supply was 1-5 volts/cm of the tank length. The run was stopped after about 30 min and the gel was transferred to UV cabinet.

The gel was photographed by a gel documentation system and the data was analyzed through computer software.

**Result and discussion:**

 **Quantification of aflatoxins by HPLC:**

***Intra-lab validation:***

The results of mycotoxins assay validation were illustrated in table (1), showing that the used method with high accuracy and extremely precise method as the Intra-day precision (RSD %) not exceed 1.81% (< 2% that recommended by **USP, 2017**) and the inter-day precision not exceed 2.63% (< 6% that recommended by **ICH, 2005**).

There were high resolution of chromatograms, it is showed in the results as the retention time of Afla -G2, -G1, -B2, -B1 and OTA standards at 0.803, 1.002, 1.901, 2.713 and 7.803 min (fig. 1).

*Selectivity and specificity:* As shown in fig (1& 2), HPLC Chromatograms of mycotoxins pure standards and spiked matrix at different levels, showing no matrix interferences were observed on the chromatograms and no interfering peaks were obtained with the same retention times (RT) of mycotoxins peaks.

Table (4): Validation sheet of mycotoxins

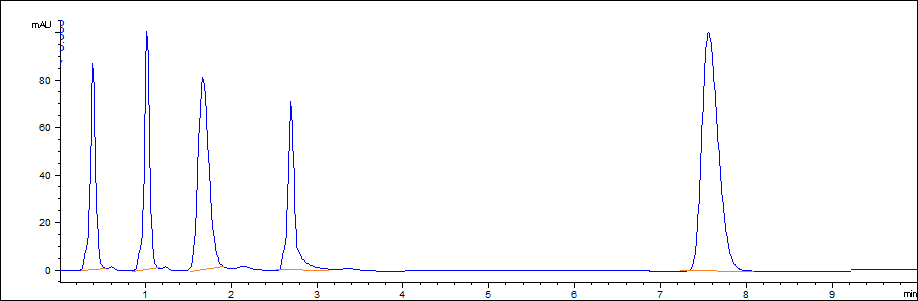
|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Parameter** | **AFB1** | **AFB2** | **AFG1** | **AFG2** | **OTA** |
| **Retention time**  **(min.)** | 2.713 | 1.901 | 1.002 | 0.803 | 7.803 |
| **Range (ppb)** | 0.015-0.3 | | | | |
| **Regression equation** | y=11826x-26.56 | y = 8822.8x-38.892 | y = 2406.3x-10.79 | y = 7790.8x-60.235 | y = 8845.1x - 9.9861 |
| **Correlation coefficient (R2)** | 0.9985 | 0.9961 | 0.9954 | 0.9995 | 0.9999 |
| **Slope (a)** | 11826 | 8822.8 | 2406.3 | 7710.7 | 8845.1 |
| **Intercept (b)** | 26.555 | 38.892 | 10.788 | 60.235 | 9.9861 |
| **LOD** | 0.002 | 0.014 | 0.015 | 0.014 | 0.008 |
| **LOQ** | 0.005 | 0.041 | 0.045 | 0.043 | 0.022 |
| **Accuracy** | 99.9 ± 0.611 | 99.8 ± 0.6 | 98.8 ± 0.47 | 96.4 ± 0.17 | 100.1 ± 0.11 |
| **Recovery %** | 98.7 | 92.2 | 95.9 | 101.82 | 100.07 |
| **Intra-day precision**  **(RSD%)** | 0.07 | 0.45 | 0.23 | 1.81 | 0.046 |
| **Inter-day precision**  **(RSD%)** | 0.34 | 1.63 | 0.73 | 2.63 | 0.23 |

Table (5): Results of mycotoxins residues in positive chicken products samples n= 30

|  |  |  |  |
| --- | --- | --- | --- |
| **Mycotoxin** | **Type of sample** | **Positive samples** | **Mean ± SE (µg/kg)** |
| Afla B1 | Nuggets | 3 | 5.03 ± 0.65 |
| Liver | 3 | 16.3 ± 0.85 |
| Thigh | 3 | 4.95 ± 0.84 |
| Afla B2 | Nuggets | 3 | 1.2 ± 0.34 |
| Liver | 3 | 10.1 ± 0.99 |
| Thigh | 3 | 0.84 ± 0.11 |
| Afla G1 | Nuggets | 5 | 0.28±0.04 |
| Liver | 3 | 1.58 ± 0.24 |
| Thigh | 3 | 0.24 ± 0.05 |
| Afla G2 | Nuggets | 3 | 0.06 ± 0.01 |
| Liver | 3 | 0.56 ± 0.09 |
| Thigh | ND | ND |

ND: not detected LOD (AFG2)= 0.014 ppb

N= samples number



G2

G1

B2

B1

Fig. (1): chromatogram of AF- G2, -G1, -B2 and-B1 standards at a conc. of (0.03, 0.075, 0.02 and 0.015 ppb; respectively)

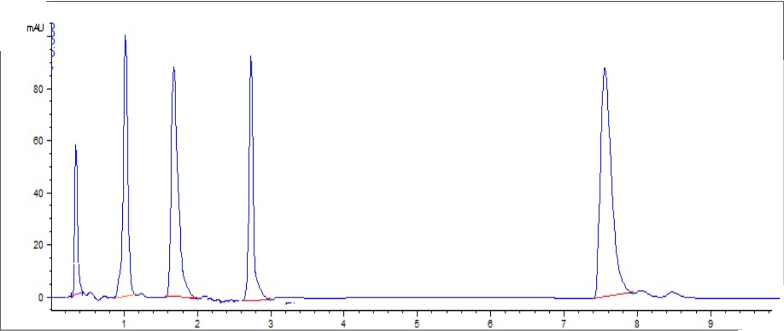


Fig. (2): chromatogram showing Afla -G2, -G1, -B2, -B1 and OTA spiked matrix at a conc. of (0.015, 0.06, 0.03, 0.03 and 0.02 ppb; respectively)

4000

3000

B1

y = 11826x - 26.555

R² = 0.9985

2000

1000

0

0 0.05 0.1 0.15 0.2 0.25 0.3 0.35

Fig. (3): Standard curve of AFB1

3000

2500

2000

1500

1000

500

0

B2

y = 8822.8x - 38.892

R² = 0.9961

0

Fig. (4): Standard curve of

***Mycotoxins residues in different chicken products:***

Due to the toxicity and carcinogenicity impact of mycotoxins residues on human public health; our study includes detecting the residues on mycotoxins (Aflatoxins types; B1, B2, G1, G 2) and Ocratoxin A (OTA) in different chicken products samples

The data represented in table (2) showing Incidence of mycotoxins in different chicken products samples showed that the level of Afla B1, Afla B2 and Afla G2 were 10 % for each and of Afla G1 and Ochra A were ranged from 10 to 16.7% for each.

Table (6): Incidence of mycotoxins residues in chicken products samples

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Mycotoxin** | **Type of sample** | **Samples no.** | **Positive samples** | **Percentage (%)** |
| **Afla B1** | Chicken wings | 30 | - | 0 |
| Chicken nuggets | 30 | 3 | 10 |
| Chicken liver | 30 | 3 | 10 |
| Chicken thigh | 30 | 3 | 10 |
| **Afla B2** | Chicken wings | 30 | - | 0 |
| Chicken nuggets | 30 | 3 | 10 |
| Chicken liver | 30 | 3 | 10 |
| Chicken thigh | 30 | 3 | 10 |
| **Afla G1** | Chicken wings | 30 | - | 0 |
| Chicken nuggets | 30 | 5 | 16.7 |
| Chicken liver | 30 | 3 | 10 |
| Chicken thigh | 30 | 3 | 10 |
| **Afla G2** | Chicken wings | 30 | - | 0 |
| Chicken nuggets | 30 | 3 | 10 |
| Chicken liver | 30 | 3 | 10 |
| Chicken thigh | 30 | - | 0 |
| **Ochra A** | Chicken wings | 30 | - | 0 |
| Chicken nuggets | 30 | 3 | 10 |
| Chicken liver | 30 | 5 | 16.7 |
| Chicken thigh | 30 | 3 | 10 |

Table (7): Results of mycotoxins residues in positive chicken products

samples

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Type of sample** | **Type of sample** | **No. of +ve samples** | **Mean ± SE (µg/kg)** | **Range**  **(µg/kg)** | |
| **Min.** | **Max.** |
| Afla B1 | Chicken wings | ND | ND | ND | ND |
| Chicken nuggets | 3 | 5.03 ± 0.65 | 3.96 | 6.19 |
| Chicken liver | 3 | 16.3 ± 0.85 | 15 | 17.9 |
| Chicken thigh | 3 | 4.95 ± 0.84 | 4.1 | 6.63 |
| Afla B2 | Chicken wings | ND | ND | ND | ND |
| Chicken nuggets | 3 | 1.2 ± 0.34 | 0.8 | 1.87 |
| Chicken liver | 3 | 10.1 ± 0.99 | 8.4 | 11.8 |
| Chicken thigh | 3 | 0.84 ± 0.11 | 0.68 | 1.06 |
| Afla G1 | Chicken wings | ND | ND | ND | ND |
| Chicken nuggets | 5 | 0.28 ± 0.04 | 0.16 | 0.4 |
| Chicken liver | 3 | 1.58 ± 0.24 | 1.21 | 2.02 |
| Chicken thigh | 3 | 0.24 ± 0.05 | 0.18 | 0.33 |
| Afla G2 | Chicken wings | ND | ND | ND | ND |
| Chicken nuggets | 3 | 0.06 ± 0.01 | 0.05 | 0.08 |
| Chicken liver | 3 | 0.56 ± 0.09 | 0.41 | 0.73 |
| Chicken thigh | ND | ND | ND | ND |
| Ochra A | Chicken wings | ND | ND | ND | ND |
| Chicken nuggets | 3 | 0.86 ± 0.09 | 0.69 | 0.98 |
| Chicken liver | 5 | 1.15 ± 0.12 | 0.9 | 1.6 |
| Chicken thigh | 3 | 0.5 ± 0.07 | 0.39 | 0.62 |

ND: not detected

Table (8): Distribution of mycotoxins in different chicken products

|  |  |  |  |
| --- | --- | --- | --- |
| Mycotoxins | Liver | Nuggets | Thigh |
| Mean | 5.37 | 1.34a | 1.63a |
| SE | 1.53 | 0.45 | 0.61 |

a: Significant (p < 0.05) with respect to liver results using ANOVA test

**Incidence of mycotoxins in different chickens products**

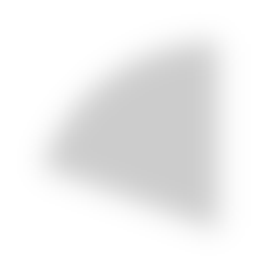
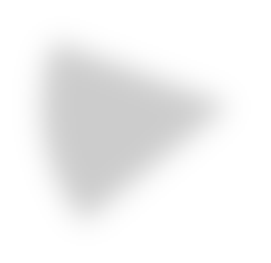
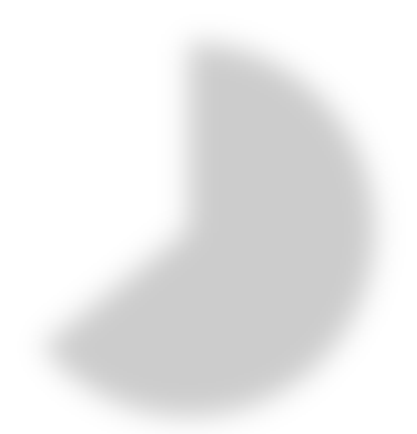
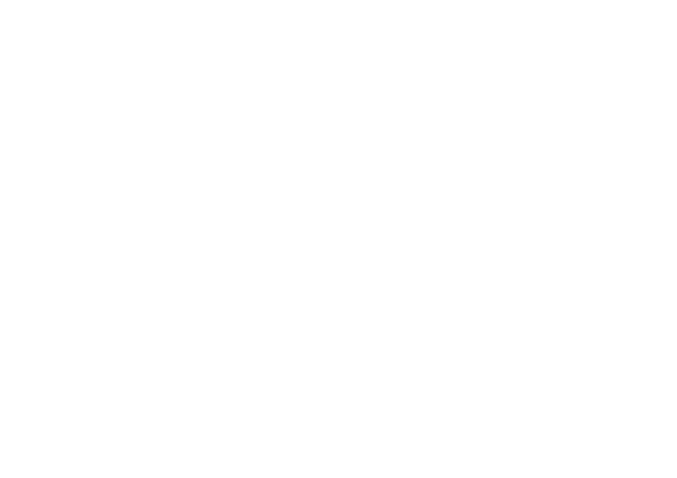
**%٢٠**

**%١٦**

**%٦٤**

Liver Nuggets Thigh

Fig. (8): Chart showing mycotoxin exist in diff. chicken products



***Mycotoxins residues in different chicken products after exposure to gamma irradiation:***

In all analyzed samples, there are a positive correlation between the increase of gamma irradiation dose applied to the samples and the level of reduction of total mycotoxins present in these samples. It is clear in table (9).

Table (9): Results of mycotoxins in different chicken products after exposure to gamma irradiation

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | **Sample type** | **(Control conc.) ppb** | **Irradiation dose (kGy)**  **ppb (Reduction %)** | | |
| **6** | **8** | **10** |
| **AFB1** | Chicken  nuggets | 6.19 | 5.97 (3.6%) | 4.97 (19.7%) | 4.1 (33.7%) |
| Chicken liver | 17.87 | 16.8 (6%) | 14.74 (17.5%) | 13.4 (25%) |
| Chicken thigh | 0.62 | 0.57 (7.5%) | 0.51 (16.5%) | 0.46 (26.6%) |
| **AFB2** | Chicken  nuggets | 1.87 | 1.74 (6.8%) | 1.3 (20%) | 1.13  (39.6%) |
| Chicken liver | 11.8 | 11.5 (6%) | 9.12 (22%) | 7.14  (39.5%) |
| Chicken thigh | 1.06 | 0.95 (10%) | 0.83 (22%) | 0.55  (48.4%) |
| **AFG1** | Chicken  nuggets | 0.4 | 0.34 (15%) | 0.24 (40%) | 0.18 (55%) |
| Chicken liver | 2.02 | 1.72 (15%) | 1.41 (30%) | 0.81 (60%) |
| Chicken thigh | 0.33 | 0.28  (15.2%) | 0.14 (42.2%) | 0.03 (90%) |
| **AFG2** | Chicken  nuggets | 0.08 | 0.07  (15.4%) | 0.043  (46.2%) | 0.01 (86%) |
| Chicken liver | 0.73 | 0.64 (12%) | 0.4 (45%) | 0.06  (91.4%) |
| **OTA** | Chicken  nuggets | 0.98 | 0.74 (25%) | 0.59 (40%) | 0.04  (96.4%) |
| Chicken liver | 1.6 | 1.2 (25%) | 0.88 (45%) | 0.64 (60%) |
| Chicken thigh | 0.62 | 0.5 (20%) | 0.25 (60%) | 0.155  (75%) |

Table (10): Total mycotoxins after gamma irradiation exposure

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Mycotoxin type** | **(Control)**  **conc. ppb ± SE** | **Irradiation dose (kGy)** | | |
| **6** | **8** | **10** |
| **AF B1** | 8.23 ± 5.08 | 7.78 ± 4.77  (5.47%) | 6.74 ± 4.2  (18.1%) | 5.99 ± 3.85  (27.22%) |
| **AF B2** | 4.91 ± 3.45 | 4.73 ± 3.92  (3.6%) | 3.75 ± 2.69  (23.63%) | 2.94 ± 2.11  (40.12%) |
| **AF G1** | 0.92 ± 0.55 | 0.78 ± 0.47  (15.22%) | 0.6 ± 0.4  (34.78%) | 0.34 ± 0.24  (63.04%) |
| **AF G2** | 0.41 ± 0.32 | 0.36 ± 0.29  (12.2%) | 0.22 ± 0.18  (46.34%) | 0.04 ± 0.02  (90.24%) |
| **OTA** | 1.07 ± 0.29 | 0.81 ± 0.2  (24.3%) | 0.57 ± 0.18  (46.73%) | 0.28 ± 0.2a  (73.83%) |
| **Total mycotoxins** | 3.3 ± 1.5 | 3.07 ± 1.4 (6.97%) | 2.53 ± 1.21 (23.33%) | 2.05 ± 1.07 (37.88%) |

a: Significant (p < 0.05) with respect to control results using ANOVA test Table

***Expression of the structure genes (nor-1) of aflatoxins by RT-PCR:***

The results showed that the DNA extract of the isolates *A.flavus* and *A.parasiticus* have the *nor-1 gene* in analyzed samples. Represented electrophoresis band pattern of Real time PCR products shown in (Fig 2).The RT-PCR results obtained with genomic DNAas a template indicated that all tested aflatoxigenic isolates expressed *nor-1* gene with significant expression levels in chicken products. Positive correlation was noted between mean expression level of *nor-1* and the amount of Aflatoxins production by isolates.

The observed differences in aflatoxins producing ability (Table 4) could be explained by differences in expression of aflatoxins biosynthetic and regulatory genes between toxigenic isolates from chicken productsdue to distinction in environmental circumstances. Transcriptional expression of the key aflatoxins biosynthetic gene nor-1 gene which encoding an enzyme catalyze the conversion of the first stable aflatoxins biosynthesis intermediate norsolorininc acid to averatin. *nor -1* gene was investigated by Real time PCR and as shown in (fig. 2)have expressed the *nor-1* gene in different levels. These result was in agreement with that obtained by **Yousefi *et al.,* 2009; Iheanacho, 2012 and Passon *et al*., 2010**who found that *A.oryzea* and *A.niger* expressedthe *nor-1* gene and with **Mayer *et al.,* 2003** who found that *A.Oryzea* expressed expressed the *nor-1* gene, and disagree with **Mahmoud, 2015** found that *A.flavus* expressed (*nor-1*) and other strains expressed a regulatory alfR. Moreover, **Cruz and Buttner, 2008** found that *A.flavus* have alfR gene and expressed it with different levels. While, **Scherm *et al.,*2005** stated that there are some other genes, alfR and alfQ present in toxigenic strains of *A.flavus* and *A.parsiticus* controlled in aflatoxin biosynthesis

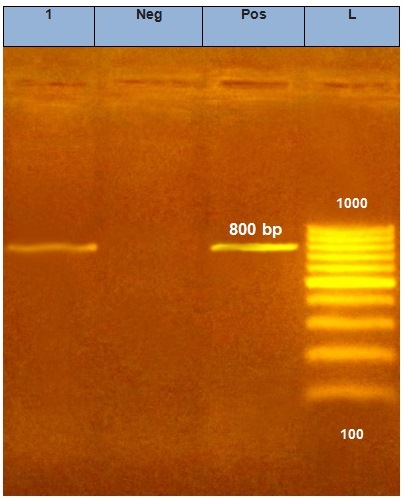


Fig. (2): Agarose gel electrophoresis of *nor-1* gene expression*,* PCR amplification product at 800 bp.

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