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Research Article

Molecular and Biological Detection of Aflatoxin B1 in Food and Food Products to Reduce Health Risk

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Abstract

Humans are exposed to aflatoxins by consuming foods contaminated with products of fungal growth. Because aflatoxins, especially aflatoxin B1, are potent carcinogens in some animals, there has beengreat interest to study the effects of long-term exposure to low levels of these important mycotoxins on humans.The main aim of the present study was to develop specific PCR and ELISA (using recombinant antibody) methods for the direct detection of aflatoxin B1 in contaminated food and food products.In this study, different food samples were collected from the Egyptian market and Aflatoxin B1 was determined by HPLC and by VICAM's monoclonal antibodies based commercial strips. Results showed that 78% of collected food samples were contaminated by Aflatoxin B1. The contaminated samples were subjected to detectionby specific PCR of the afiR gene (aflatoxin B1 gene) and the PCR results confirmed the results observed by the two previous

methods. The recombinant protein resulting from the *in vitro* transcribed afiR gene (Egyptian isolate) has been used to produce recombinant protein antiserum that was employed to develop ELISA and was successful to detect the Aflatoxin B1 in the contaminated samples with low dilution. This high level of test sensitivity was suggested to be due to the low molecular weight of the recombinant antiserum used. In conclusion: biochemical and molecular analyses of gene (s) responsible for aflatoxins synthesis is of great interest to assure food safety by detecting and preventing mycotoxin risk exposure. In future studies, enlargement of the recombinant protein by different methods would be recommended and this will increase its sensitivity. Further surveys are highly recommended in order to establish a database for mycotoxin occurrence in Egypt to minimize the possible health risks in animals and humans.

Introduction

Aflatoxins (AF) are a group of toxics, mutagenic, teratogenic and carcinogenic fungal metabolites [1,2]. They are secondary metabolites produced mainly by different strains of *Aspergillus fluvus, Aspergilllius parasiticus* [3] and *Aspergillus nomius* that pollute different kinds of food and feed during growth, processing, storage and transportation. Nowadays, about 20 types of different aflatoxins are known and classified into B1, B2, G1, G2, M1 and M2 as metabolites in serum of animals and humans, based on their chemical structure, chromatographic and fluorescence's profiles [4,5]. AFB1 is the most toxic aflatoxin and is a common contaminant of many foods, mostly representing 75% of aflatoxins contaminated food and it acts as mutagens and carcinogens to man [2,6]

Growth and production suppression, immunosuppression and liver enzymes disorders are the most common symptoms result from the AF contamination [7,8]. AFs are mycotoxins group and are potent mutagenic, carcinogenic, teratogenic, hepatotoxic and immunosuppressive agents [9]. Aflatoxin M1 is a derivative of Aflatoxin B1 and is a hepatocarcinogenic, formed in liver and excreted into the milk formed by the mammary glands of both human and lactating animals that have been fed with AFB1 contaminated diet. Although AFM1 is about ten times less toxigenic than AFB1, its cytotoxic and carcinogenic impacts have been demonstrated in several species [10,11].

International Agency for Research on Cancer [12] has put AFM1 into group 1 carcinogenic for humans. AFM1 is preferentially linked to milk casein fraction and this could result in dairy products contamination at higher AFM1 concentration than the original milk [13,14].

It is important to develop new methodologies and systems able to quantify the aflatoxins concentrations that satisfy the restrictions proposedby the organizations in charge of controlling these compounds. Accordingly, several techniqueshave been employed to do this, such as: chromatography especially the most commonly used HPLC technique with immunoaffinity clean up column have been used [15] for aflatoxin detection; however, they are expensive, time-consuming, and require many skills. Other methods include tests that utilize the proven sensitivity and selectivity of VICAM's monoclonal antibodies to accurately detect and measure total aflatoxins B1, B2, G1, and G2 at levels less than 2 ppb and as high as 100 ppb. [VICAM, Milford, MA, USA].

Other immunological methods mostly based on ELISA that has a good sensitivity, speed and simplicity have been used [16,17]. Immunoassay methods usually use 3 types antibodies: monoclonal antibodies (mAbs), polyclonal antibodies(pAbs), and recombinant antibodies (rAbs). rAbs are generally produced by expressing an antibody gene in a prokaryotic or eukaryotic organism bacterial, yeast, or mammalian cells). This allows the creation of antibody libraries, which are then used to select antibodies with desired characteristics. Similarly, functional fragments of antibodies have been obtained from intact antibodies, or through recombinant DNA technology. After PCR amplification, the antibody fragment DNA is ligated into a plasmid or a phage, and then, itis expressed in a host cell. The most used antibody fragments include single-chain variable fragments (scFvs), which comprise the variable parts (light (VL) and heavy (VH) chains), and single-chain Fab antibody (scFab) made of light chain (LC) and fragment difficult (Fd). In addition, their variants have been produced including single domain antibodies, diabodies, and tribodies [18,19].

PCR-based methods have emerged as major tools for detection of aflatoxin-producing fungi in foods [7] and real-time PCR has been used in order to achieve the development of rapid, simple, highly sensitive and low-cost techniques.

Furthermore, many scientists are keen to discover the contaminated food directly by using specific genes for specific fungus and the functions genes that control the pathway of aflatoxins synthesis by the fungi. Gallo-Fernández *et al*. [20] tested the presence of these genes in the genome of the aflatoxins producing fungi. The main aim of the present study was to develop specific PCR and ELISA (using recombinant antibody) methods for the direct detection of aflatoxin B1 in contaminated food and food products from the Egyptian market.

Methods

Sample Collection

Food samples were collected from products of three different companies from the Egyptian market. 12packages from three companies; four companies; four packages form each company (0.5kg/package) were collected. The same was applied for pea nuts (300g/package) and wheat flour (2kg/package) that were collected and subjected to different analysis and detection of aflatoxin B1.

Methods used for AFB1 Detection in the Collected Samples

HPLC

AFB1 was extracted from the collected food samples and residual Aflatoxin (B1,B2,G1,G2) were analyzed using HPLC. One ml of each sample was centrifuged at 6000 rpm for 15 min then filtered through a 0.45µm hydrophobic polytetrafluoroethylene syringe filter prior to Gel Pours Chromatography (GPC) analysis. The supernatant was transferred to 1.5 ml micro-tube that was passed through an immunoaffinity column at a rate of 1-2 drops/second. The column was washed with 10 ml of 90:10 water: methanol twice at a flow rate of 3ml/min, then aflatoxins were eluted by slowly passing 1 ml of methanol through the column. In cases where the eluent was not clear, it was repassed through a 0.45µm filter [21]. Then, 100µl trifluoracetic acid

plus 200µl n-hexane were added to samples residue, followed by vortex mixing for 30 seconds and the vial was left for 15 min, then 900µl (Water: Acetonitrile, 9:1) were added and vortex-mixed. Hexane layer was removed and samples were ready for HPLC analysis. AFS were determined according to the method of Scaglioni and Badiale-Furlong [22], using Waters HPLC system, Model 6000, a solvent delivery system, and Model 720 system controller equipped with Fluorescence detector (Model 274) at 360 EX, and 450 EM. The separation was achieved with a Waters symmetry column, (150x 4.6 mm i.d), 5µm at a flow rate of 1ml /min with an isocratic system composed of 1% Acetic acid: Methanol: Acetonitrile (55: 35:10).

Afla-(Vt)

The samples were extracted according to the procedure Afla-Vt [VICAM, Milford, MA, USA]. Afla-V strip tests utilize the proven sensitivity and selectivity of VICAM's monoclonal antibodies to accurately detect and measure total aflatoxins B1, B2, G1, and G2 at levels as low as 2 ppb and as high as 100ppb.(2ug/kg to 100ug/kg) [VICAM, Milford, MA, USA].

Molecular Detection using Specific (PCR)

DNA Extraction from the Collected (Samples)

The collected food samples were subjected to DNA extraction using QiaGene DNA extraction kit (QiaGene, Germany). The extracted DNA was dissolved in DEPC-treated water, quantified spectrophotometrically and analyzed using 1.2% agarose gel.

Specific PCR (reaction)

The aflR gene was amplified using specific PCR (744bP) in a reaction consisted of; 1 µl of DNA added to 2.5 µl Taq polymerase buffer 10x (Promega, USA) containing a final concentration of 1 mM MgCl2, 0.2 Mm dNTPs, 20 pmol of each primer: Forward primer; 5`-TAAGCAGAATTCGAATAGCTTCGCAGGGTGGT-`3 and the Reverse primer: is 5`-GAATAGCTTCGCAGGGTGGTGCGGCCGCTAAGCA-`3(primers were designed by primer Blast, NCBI) and 0.2µl Taq polymerase (5 U/ µl) in a final reaction volume of 25 µl. The PCR reaction program started with initial denaturation at 95°C for two minutes followed by 35 cycles. Each cycle programmed with 95°C for 2 minutes, 58°C for one minute and 72°C for one minute. At the end of the last cycle, a final extension step at 72°C for 5 minutes was added. The PCR amplification products were electrophoresed in 1.5% agarose with 0.5X TBE buffer and visually analyzed by a gel documentation system (Syngene).

Cloning, Sequencing and Sequence Analysis and (Subcloning)

The resultant PCR product was excised from the gel and purified using a QIA quick gel extraction kit (Qiagen Inc., Germany). Purified DNAs were ligated into the pGEM-T vector (Promega Co., USA). Recombinant plasmids were then directly sequenced using automated sequencer (Macrogene Company, Korea), with vector universal primer. DNA homology searches were carried out with the NCB1 data bases,

using the BLAST network service. *EcoRI* and *NotI* restriction enzymes were used for gene release and insertion into pPROEXHTa expression vector (Life Technologies, USA), and the recombinant plasmids were transformed into the competent *E. coli* (BL21) cells and the recombinant protein was obtained.

Recombinant Protein and SDS (PAGE)

The recombinant protein was separated on SDS PAGE 12% and the molecular size was determined by using the protein standard marker low range (BioRad, USA). The gel preparation, staining and destaining was performed according to Laemmli [23].

Epitope Prediction and Antigenic Determination of the Recombinant Protein

To examine the epitope in different types of antigenic determinants that could be recognized by the immune systems, B-cell epitope prediction analysis was performed according to Kolaskar&Tongaonkar [24].

Immunization and Production of Antibodies

The purified protein (1mg/ml) was mixed with an equal volume of Freund's complete adjuvant (Sigma) and injected into New Zealand white rabbit (weight ~ 4kg). At first and second weeks, animals were intravenously and subcutaneously injected with 500µl and 1ml, respectively. Then, next 3-weekly injections were given subcutaneously with 1ml Freund's incomplete adjuvant (Sigma) and purified protein (1:1). Two weeks later; one injection with 500µg of the purified protein was administered intravenously. Rabbit's blood samples were collected; left 2hrs at room temperature to clot and stored overnight at 4°C. The separated antiserum was clarified by centrifugation at 3000 rpm for 10 min and mixed with equal volume of glycerol and then divided into aliquots and stored at -20°C.

Serum IgGs Purification and Fractionation

Different rabbits' sera were obtained by centrifugation of immunized rabbits' blood at 4000 rpm for 5 min at 4°C. IgG fractions were obtained by loading sera on affinity Protein G-Sepharose column. In brief, IgG1 fraction was eluted with glycine buffer, pH 2.7 and IgG3 fraction was obtained by elution from the Protein G column with glycine buffer, pH 3.5. All fractions of IgGs were immediately neutralized by neutralization buffer (1 M Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA).

Labelling of Antibodies with Alkaline Phosphatase

Ten milligrams of alkaline phosphatase (AP) were mixed with the purified IgGs (2.5mg) in 5ml of 50 mM phosphate buffer, pH 6.8. The mixtures of (IgG-AP) was dialyzed against 2L of 50 mM phosphate buffer for 24 h at 4°C. After adding 1.0ml of 1.25% glutaraldehyde solution to each mixture, the solution was stirred gently for 2 h at room temperature. 250µl of 0.2M glycine solution were added to the mixture by further stirring for 2 h. Then, the mixtures were dialyzed two times against 2 L 1.0×PBS containing 1mM magnesium chloride, followed by centrifugation for 5 min at 10,000 rpm to remove any precipitate. For

further purification of all conjugates, each conjugate was applied to Sephacryl S200 column (5 × 150mm, GE Health care, Sweden) previously equilibrated with PBS and eluted with the same buffer.

Indirect Enzyme-Linked Immunosorbent Assay (I-ELISA)

Indirect ELISA was used to test the effectiveness of the produced polyclonal antibodies as detector for aflatoxin B1 existence in food samples. The results were compared with an antiserum produced by Sigma (Germany). One gm of contaminated food samples was extracted in 10ml coating buffer. The extracts (100µl/well) were incubated overnight at 4˚C and blocked with 200µl of blocking buffer (1X PBS and 0.5% BSA) for 1hr. at room temperature. For each well, 100µl of the extracted sample were added and incubated at 37˚C for 3 hrs. Then, 100µl of diluted secondary antibody-alkaline phosphatase conjugate (anti-rabbit antibody) were added and incubated for 1 hr. at 37˚C. All washing steps between incubations were performed with 1 X PBS-T buffer. Finally, freshly prepared PNPP substrate was added; incubated for 30min at room temperature away from direct light and the absorbance was measured at 405nm.

Results

The Aflatoxin B1 Detection in the Collected Food Samples Using HPLC and VICAM

The data presented in table 1 revealed the presence of Aflatoxin B1 in all examined samples. The HPLC analysis results were closely similar to that obtained byAfla-Vt [VICAM, Milford, MA, USA]. The sensitivity of Afla-Vt test wasn`t as high as that of our HPLC analysis. Results showed that 78% of collected food samples were contaminated by Aflatoxin B1.

Table 1: *The two biological methods used for aflatoxin B1 detection in the contaminated food samples*

Samples	HPLC	VICAM
Peanut 1	+++	+++
Peanut 2	+++	++
Peanut 3	+++	+++
Flour 1	++	++
Flour 2	+++	++
Flour 3	++	+
Milk-powder 1	++	++
Milk-powder 2	+	-
Milk-powder 3	++	+
Contaminated flour with AFB1 (50 ng).	+++	+++
Contaminated flour with AFB1 (75 ng).	+++	++
Contaminated flour with AFB1 (100 ng).	+++	+++

Molecular Detection of the Aflatoxin B1 in Food Samples Using Specific PCR

In the present study, the 39 food samples were subjected to DNA extraction and specific PCR using the specific primers of the Aflatoxin B1 gene and the data showed that, a unique band with molecular size about 760 bp was observed in 36 samples (Fig. 1A). The negative amplification was observed in the flour which contaminated by Aflatoxin (50, 75 and 100mg) in a respective manner. The amplified DNA was cloned and the *in vitro* transcribed protein was separated on SDS-PAGE (Fig. 1B). Data presented in figure (1B) revealed that a protein with molecular size about 28kDa was observed.

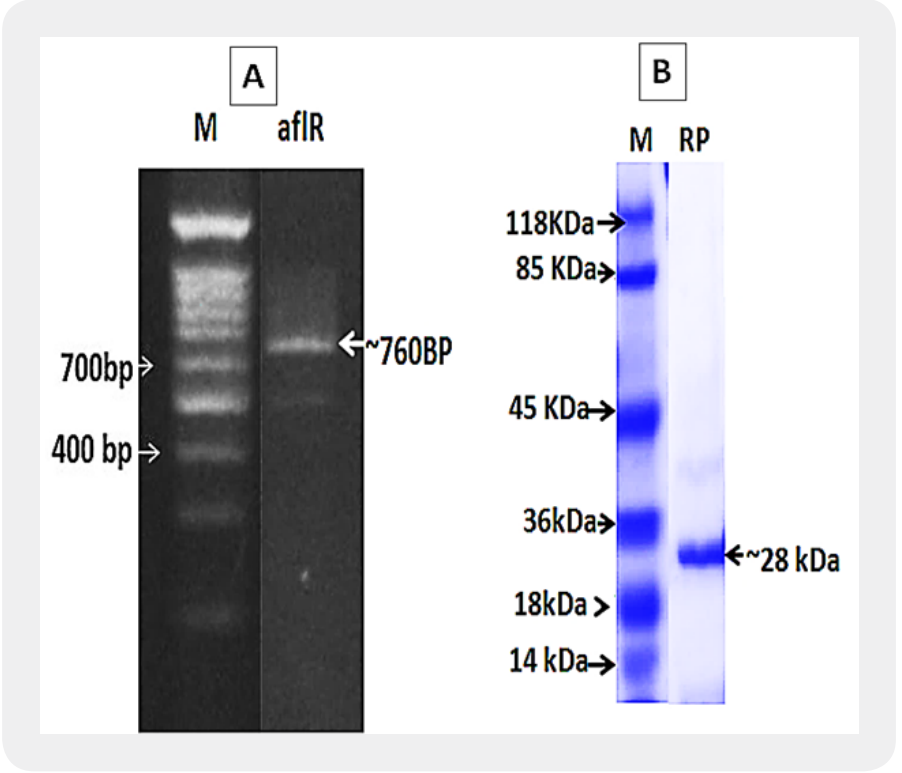

Figure 1: *A: PCR product amplified using the specific primers of the afiR gene (Aflatoxin B1). DNA marker and afiR the amplified gene in molecular size about 760bp. B: The recombinant protein of the in vitro transcribed afiR gene (Aflatoxin B1) with molecular size about 28kDa*

Sequencing and Sequence Analysis and Phylogenic Construction

The PCR product was purified and subjected to DNA sequence and about 750 nucleotides were obtained The sequence was aligned using the NCBI analysis tool and the results showed similarity with 97% with the other afiR genes listed on Gene Bank. The sequence was compared with 50 sequences of different of afiR genes already listed on Gene bank AND were used to construct the phylogenetic tree and it was observed that Egyptian afiR gene was closely similar to afiR gene MH752587 which isolated from *Aspergillus* sp. PS-2018c isolate BN038G AFLR, Arizona, USA (Fig.2).

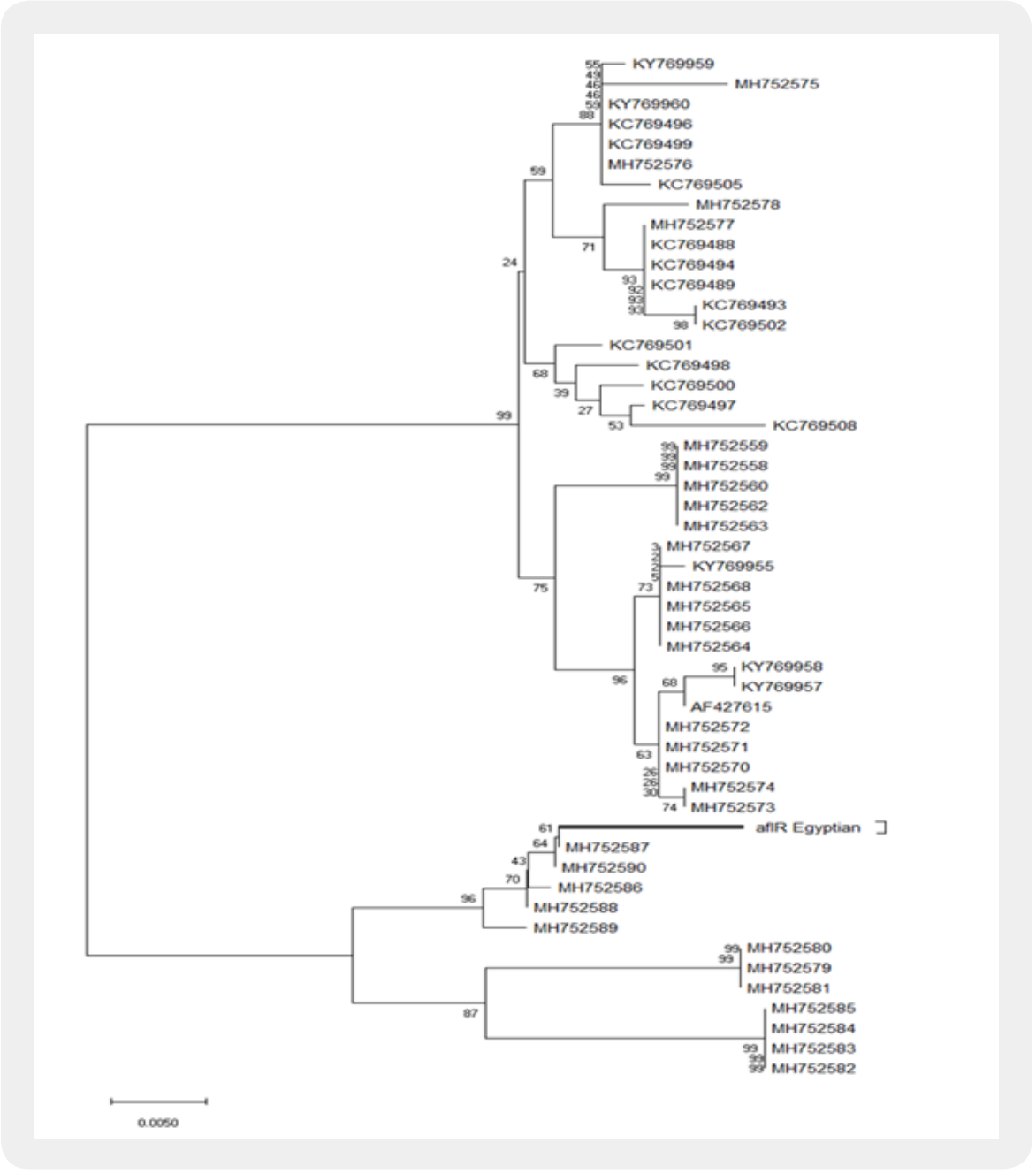

Figure 2: *Phylogenetic tree for the amplified aflatoxin B1 based on the DNA nucleotide sequence and compared with the other 50 AFB1genes listed on gene bank. The phylogeny was constructed using Mega 6 program.*

Kolaskar&Tongaonkar Antigenicity Test

The total number of the deduced amino acids of the obtained gene was found to be 256 amino acids. The results obtained by Kolaskar&Tongaonkar [24] antigenicity test presented in figure 2 revealed that 8

peptides showed antigenic activity. The length of these peptides ranged from 8 to 14 amino acids. Their positions started from the amino acids number; 26, 66, 107,136, 170, 186. 205 and 236, respectively as shown in figure-3.

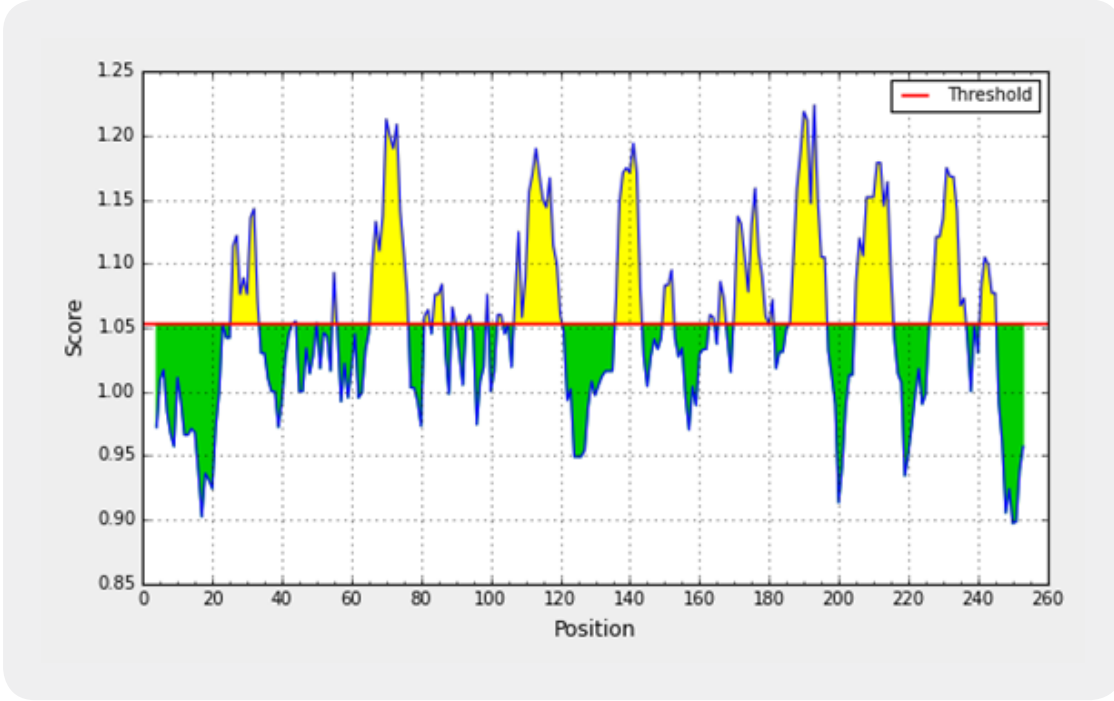

Figure 3: *The possible antigenic activity of the recombinant protein (afiR) produced in this study*

Table 2: *Predicted peptides, their length and position*

No.	Start	End	Peptide	Length	>aflR deduced amino acid sequence
1	26	33	LMQVPKIY	8	MSHSYNTFAGWPINTPTGRTQGSLALMQVPKI
2	66	76	EHYLLFLVQFV	11	YLAGNKSFLGSQPAHDGLRYLEPEACMRAGQL
3	107	120	TPQLVTFVYIHLDL	14	AEHYLLFLVQFVNRSRSSLVTRFQPRYVNKEC
4	136	143	FTLCVFFP	8	TARQSLGQVRTPQLVTFVYIHLDLSARQRKGQ
5	170	179	PGRCVPPRLA	10	ATLQEKAPTLCVPPPAPNSKLYSIPSSRPPRW
6	186	196	LAVRVVPVQKC	11	LTIFPPGHLPGRCVPPRLAALESSGIAVRVVPVQKC
7	205	215	VLGVSNVVLPV	11	DAPRRNRPVLGVSNVVLPVNTWSPSGWAAI
8	227	236	RALPVPLIQL	10	RALPVPLIQLGDHQRVFLQPDRNRDIRRIT

Recombinant Protein and Antiserum Purification

Serum obtained from the immunized rabbits were fractionated using affinity chromatography protein G-Sepharose column and one band of the conventional IgG with molecular weight of 130 kDa was observed. Also, two bands of heavy chain with molecular weight of 42kDa and the other light chain with a molecular weight of 19kDa under reducing condition.

Detection of the Aflatoxin B1 in the Food Samples Using the Recombinant Antiserum

In this study, the recombinant antiserum was able to detect the presence of the Aflatoxin B1 protein in food samples contaminated with the Aflatoxin B1 (Table 3).

Table 3: *Comparative results obtained by specific PCR and the recombinant antiserum*

Samples	Specific PCR	Recombinant Anti-serum
Peanut 1	+	+++
Peanut 2	+	+++
Peanut 3	+	++
Flour 1	+	++
Flour 2	+	+++
Flour 3	+	++
Milk-powder 1	+	+++
Milk-powder 2	+	++
Milk-powder 3	+	++
Contaminated flour with AFB1 (50 ng).	+	-
Contaminated flour with AFB1 (100 ng).	+	-
Contaminated flour with AFB1 (200 ng).	+	++

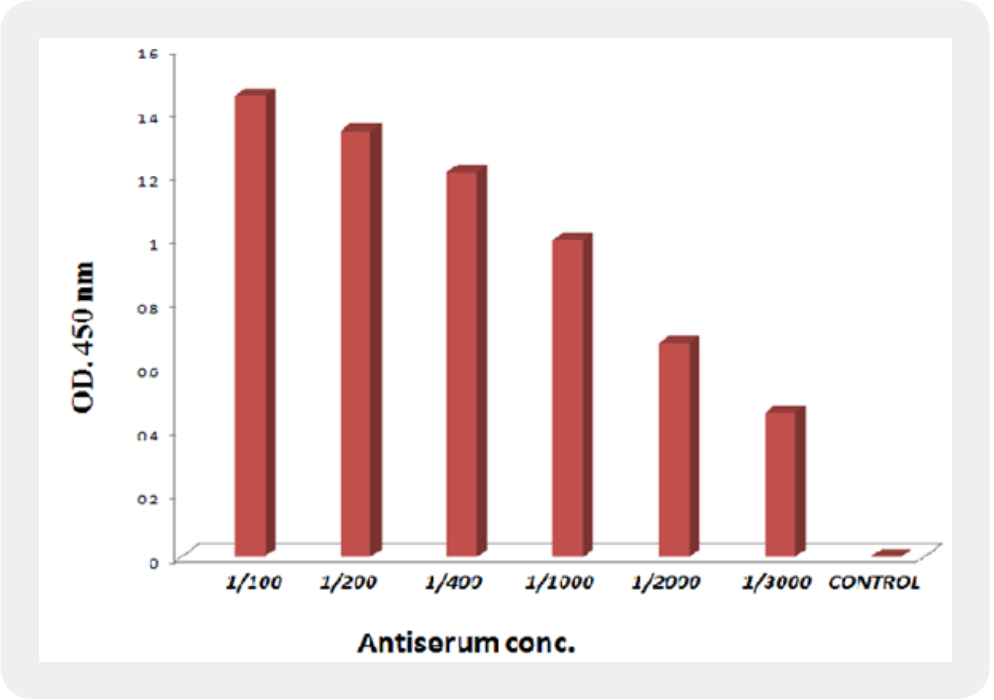

Figure 4: *The Indirect ELISA sensitivity against Aflatoxin B1in contaminated food samples using the recombinant antiserum*

Discussion

Contamination of food and food products with mycotoxins represents a serious health issue for animals and humans in developing countries. Mycotoxins are naturally occurring ones which are formed by the presence of moulds in food stuffs and cannot be completely controlled. These mycotoxins are known to present a health risk for both animals and humans, as these toxins are stable and can withstand the most severe process, particularly the most toxic AFB1 [25,26]. So, these toxins can represent a problem in food and processed foods and may lead to health hazards. Therefore, the main aim of the present study was to develop PCR and ELISA methods for the direct detection of AFB1 in different food and food products samples that are familiar to people in Egypt market.

PCR-based methods have emerged as major tools for detection of aflatoxin-producing fungi in foods [7,27] and real-time PCR has been used in order to achieve the development of rapid, simple, highly sensitive and low-cost techniques.

In the present study, the 39 food samples were subjected to DNA extraction and specific PCR using the specific primers of the Aflatoxin B1 gene and the data showed that, a unique band with molecular size about 760 bp was observed in 36 samples (Fig. 1A). The negative amplification was observed in the flour which contaminated by Aflatoxin (50, 75 and 100mg) in a respective manner. The amplified DNA was cloned and the *in vitro* transcribed protein was separated on SDS-PAGE (Fig. 1B). Data presented in figure (1B) revealed that a protein with molecular size about 28kDa was observed.

The PCR product was purified and subjected to DNA sequence and about 750 nucleotides were obtained. The sequence was aligned using the NCBI analysis tool and the results showed similarity with 97% with the other afiR genes listed on Gene Bank. The sequence was compared with 50 sequences of different of afiR genes already listed on Gene bank and were used to construct the phylogenetic tree and it was observed that Egyptian afiR gene was closely similar to afiR gene MH752587 which isolated from Aspergillus sp. PS-2018c isolate BN038G AFLR, Arizona, USA (Fig.2).

ELISA methods have a good sensitivity, speed and simplicity and have been used for the detection of aflatoxin B1[16,17]. These methods usually use 3 types antibodies: monoclonal antibodies (mAbs), polyclonal antibodies (pAbs), and recombinant antibodies (rAbs). rAbs are generally produced by ex-pressing an antibody gene in a prokaryotic or eukaryotic organism bacterial, yeast, or mammalian cells). This allows the creation of antibody libraries, which are then used to select antibodies with desired characteristics. Similarly, functional fragments of antibodies have been obtained from intact antibodies, or through recombinant DNA technology. After PCR amplification, the antibody fragment DNA is ligated into a plasmid or a phage, and then, it is expressed in a host cell.

The total number of the deduced amino acids of the obtained gene was found to be 256 amino acids. The results obtained by Kolaskar & Tongaonkar [24] antigenicity test presented in figure-2 revealed that 8 peptides showed antigenic activity. The length of these peptides ranged from 8 to 14 amino acids. Their positions started from the amino acids number; 26, 66, 107,136, 170, 186. 205 and 236, respectively as shown in figure-3.

Serum obtained from the immunized rabbits were fractionated using affinity chromatography protein G-Sepharose column and one band of the conventional IgG with molecular weight of 130 kDa was observed. Also, two bands of heavy chain with molecular weight of 42kDa and the other light chain with a molecular weight of 19kDa under reducing condition. Glutaraldehyde was used to prepare the conjugates using a ratio of 4:1 of IgG and the enzymes (AP). The obtained IgG-AP conjugate was purified by gel filtration Sephacryl S200 column.

ELISA was found to be indecisive in distinguishing between different antigens, due to the presence of common epitopes in the protein surface. In this study, the recombinant antiserum was able to detect the presence of the Aflatoxin B1 protein in food samples contaminated with the Aflatoxin B1 (Table 3). The results obtained by the produced recombinant antiserum showed a high level of sensitivity because the low molecular weight of the recombinant protein.

Conclusions

This study revealed the occurrence of AFB1 in some food and food products collected from the Egyptian market using four different analytical methods: HPLC, VICAM's monoclonal antibodies based commercial strips, PCR and ELISA employing recombinant antibody. The use of recombinant antibody in ELISA showed the highest level of sensitivity. In the future study, the recombinant protein should be enlarged by different methods, and this will further increase its sensitivity. There should be biochemical and molecular

analysis of gene(s) responsible for aflatoxins synthesis to assure food safety by detecting and preventing mycotoxin risk exposure. Further surveys are highly recommended in order to establish a database for mycotoxin occurrence in Egypt to minimize the possible health risks in animals and humans.

Acknowledgements

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Conflict of Interest

The authors declare no conflicts of interest.

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