PCR Detection of *Aspergillus flavus* Isolates for Aflatoxin B1 producer

تشخيص عزلات للفطر *Aspergillus flavus* المنتجه للافلاتوكسين B1 بأستخدام التفاعل التسلسلي لبلمرة الدنا

Abdulkareem Jasim Hashim Abdulkareem A. Al-Kazaz
Hadeel Waleed Abdulmalek
Biotechnology Dept/ College of Science/ University of Baghdad
عبدالكريم جاسم هاشم عبدالكريم عبدالرزاق عبدالوهاب هديل وليد عبدالملك
جامعة بغداد/ كلية العلوم / قسم التقنيات الاحيائية

Abstract

The ability of five *Aspergillus flavus* that produce Aflatoxin B1 have been detected using coconut medium as substrate. Chromatographical analysis by TLC and HPLC revealed that, three out of five isolates were a good producer for the Aflatoxin B1. In this study, rapid assessment of five isolates of *A. flavus* was accomplished using an indigenously designed primer pair for the Aflatoxin regulatory gene *aflR* in polymerase chain reaction (PCR). Specificity was assayed in pure culture systems using DNA extracted from five different *A. flavus* isolates as PCR template. Positive amplification was achieved only with DNA from *A. flavus* that produce Aflatoxin B1.

المستخلص

هدفت الدراسه الى الكشف عن قابلية عزلات *Aspergillus flavus* لانتاج الافلاتوكسين B1 باستخدام جوز الهند كوسط زرعي بينت نتائج التحليل باستخدام تقنية الكروموتوغرافيه الطبقة الرقيقة و كروموتوغرافي السائل العالي الاداء ان ثلاث عزلات من بين خمسه كانت منتجه للافلاتوكسين B1. تم في هذه الدراسه الكشف السريع عن انتاج الافلاتوكسين B1 من العزلات المحليه الخمسه للفطر *A. flavus* و ذلك باستخدام زوجين من البوادئ متخصصه *afl.R2* , *afl.R1* لتحديد الجين المنظم لانتاج الافلاتوكسين *aflR* في تفاعل البلمره المتسلسل (PCR) باستخدام الدنا المستخلص من العزلات الخمسه كقالب.

Introduction

Aflatoxins are secondary metabolites produced by the aflatoxigenic fungi *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. bombycis* and *A. pseudotamarii* [1]. *A. flavus* mainly infect maize, cotton, peanuts, tree nuts [2], figs [3] and spices [4–5]. The contamination of foods by aflatoxigenic fungi, especially in tropical countries may occur during preharvesting, processing, transportation and storage [2]. Different methods are implemented to screen the ability of Aflatoxins production of *Aspergillus* species. These methods commonly use the culture of strains in suitable liquid or solid media. For this purpose many media are used: Yeast extract-sucrose (YES) [6], Reddy medium, and natural media with wheat, rice, peanut, malt, date, palm kernel or coconut extracts [7, 8, 9, and 10]. Identification of mycotoxigenic fungal contamination prerequisite for Aflatoxin avoidance. The detection of the aflatoxigenic fungi is usually performed by traditional dilution plating, use of diagnostic media or by immunological methods. The traditional methods are time consuming, labour-intensive, costly, require mycological expertise and facilities. Immunological methods and diagnostic media have limitation in identifying the aflatoxigenic fungi due to false positives. It also require extensive purification steps of samples [1,7].

Keywords: Aflatoxins, *Aspergillus flavus*, PCR, *aflR*

The polymerase chain reaction (PCR) facilitates *in vitro* amplification of the target sequence. The main advantages of PCR is that organisms need not be cultured, at least not for long, 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 [11]. Many pathogenic organisms have been detected using PCR [12]. The biosynthetic pathway for Aflatoxin production by *A. flavus* has been deciphered and genes in the Aflatoxin biosynthetic pathway have been identified [13]. The gene *aflR-2* has shown to regulate Aflatoxin biosynthesis [14]. Few other genes of the Aflatoxin biosynthetic pathway cloned and sequenced [15]. The PCR reaction was targeted Aflatoxin synthesis regulatory gene (*aflR1*) since it is conserved in *A. flavus* and *A. parasiticus* [2], indicating the possibility of detection of both the species with the same PCR system (primers/reaction). The aim of present study was to detect *A. flavus* that produce *afla* B1 using PCR technique.

Materials and methods

- Fungal isolates:

The isolates *A. flavus* 1, 2 and 5 were obtained from Department of Biotechnology/ University of Baghdad while *A. flavus* 3 and 4 were obtained from Ministry of Sciences and Technology. All fungal isolates were cultivated at 28±1°C on potato dextrose agar (PDA) and stored at 4°C.

- Culture conditions for Aflatoxin B1 production

Solid-state fermentation (SSF) medium consists of 10gm coconut has been used for Aflatoxin B1 production. The substrate was humidified with a 30 ml (1:3) w/v of distilled water. The humidified medium was placed in 250 ml Erlenmeyer flasks and autoclaved at 121°C, 15 min. The sterilized medium was inoculated with 10^5 spores from 5-day-old cultures of pure *A. flavus* isolates in duplicate, the flasks then incubated for 10 days at 28±1º C. Flasks without inoculation were used as control [16].

- Extraction, purification and detection of Aflatoxin B1

Aflatoxin B1 has been extracted from coconut cultures using chloroform 1:4 w/v. The contents of the flasks were shacked for 30min. The crud extracts were filtered through gauze, and then through Whatman No.1 filter paper. The extract has been purified according to FAO procedure with few modifications [17]**.**

Aflatoxin B1 has been detected by thin-layer chromatography (TLC) using chloroform: acetone 97:3 v/v as mobile phase. The florescence spots have been observed under UV-cabinet at 365nm and compared to standard Aflatoxin B1(Sigma) as control. The detection has been confirmed using high performance liquid chromatography (HPLC) with following conditions: column: 250x4.6mm, particle size 5 µm, ODS (C18), mobile phase acetonitril: water 40:60 v/v, flow rate 1ml/min., Detector UV-365nm.

- Extraction of fungal DNA

DNA was extracted from 0.5 g (wet weight), fungal mycelia / spores harvested from 3 days growing cultures in potato dextrose broth (PDB). The mycelium/ spores were transferred to a mortar, frozen in liquid nitrogen and were ground well. Steps of extraction had been completed using EZ-10 Spin Column Fungal Genomic DNA Mini-preps Kit, BIO BASIC INC., Markham Ontario, Canada.

- PCR Assay

PCR primers were designed using primer 3 software and were purchased from CinnaGen, Germany, CinnaGen 5X PCR Master Mix, ready to Load (Green) had been used in this work.

-Polymerase Chain Reaction

The polymerase chain reaction was used to amplify the Aflatoxin regulatory gene fragments of aflatoxigenic fungal genomic DNA. The sequence of the forward and reverse primers *aflR1* of the Aflatoxin regulatory gene was (5⁻ AACCGCATCCACAATCTCAT 3⁻) and (5⁻AGTGCAGTTCGCTCAGAACA 3⁻), The primers that cover the region from 540 to 1338 of Aflatoxin regulatory gene with product size of 798 base pairs (bp) have been patented [18].

The polymerase chain reaction was performed in 25 ml; each reaction mixture was heated to 95ºC for 10min. A total of 30 PCR cycles, each cycle at 0.3min at 94ºC for denaturation, 0.45 min at 55ºC for annealing, 1.15 min at 72ºC for extension and a 10min final extension at 72ºC. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel in (1x) TBE buffer (50 mM Tris–acetate, 1 mM EDTA, pH 8.0) stained in 0.5 mg/ml ethidium bromide

- Nested PCR

Nested PCR was carried out using the primer set *aflR2*. The sequence of the primers was 5⁻ GCACCCTGTCTTCCCTAACA 3⁻ and 5⁻ ACGACCATGCTCAGCAAGTA 3⁻ with product size of 400 bp, and is nested to the primer *aflR1*. The diluted PCR product of the primer *aflR1* was used as the template to carry out PCR using the primer *aflR2*. PCR was performed under the above mentioned conditions [19, 20].

Results and discussion

Aflatoxin B1 production and it[s] determination by TLC and HPLC

Aflatoxin production abilities tested previously by TLC under UV light at 365nm were in concordance with those obtained by HPLC determination. The three isolates of *A. flavus* 3, 4, and 5 showed fluorescence under UV light, which means that, these isolates have been, produced Aflatoxin B1 in coconut medium after 10 days of incubation at 28±1 ºC. No fluorescence of Aflatoxin B1 was detected in the extract of the isolates 1 and 2 Figure (1). Coconut is superior of other media used so far for the production of Aflatoxins, some of the factors that might account for the high yield obtained on coconut may be the nature and content of neutral fat in the mature coconut kernel. The oil contents of fresh coconut are 30 to 40 %. However, the fatty acids constituents of coconut are predominantly the C12 and C14 acids, lauric and myristic acids [16].

Fig(1):Detection of Aflatoxin B1 produced from *Aspergillus flavus* isolates by thin-layer chromatography using Chloroform:acetone 97:3 as mobile phase.
Lane 1, 2, 3, 4 and 5 are the numbers of fungal isolates.
Lane S is Standard Aflatoxin B1 (200 µg/ml methanol)

This result compared the response of producer isolates of *A. flavus* and their Aflatoxin producing ability assessed by HPLC. These findings were similar to the previous study [19] where all the blue fluorescence observed on TLC was associated with the presence of Aflatoxin B1 detected by the HPLC.

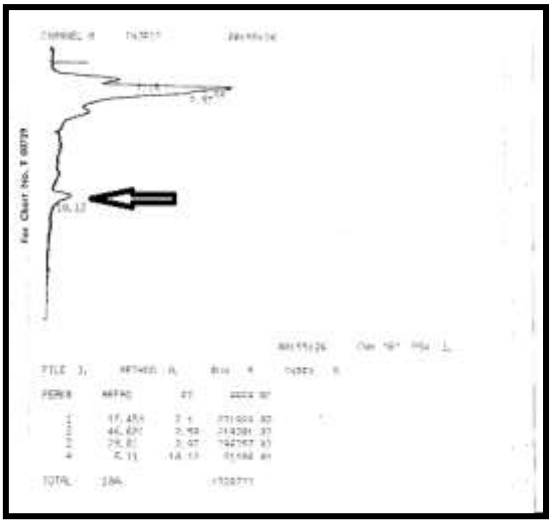
Extraction of coconut culture filtrates with chloroform followed by subsequent analysis by HPLC allowed the detection and the quantification of Aflatoxin B1 according to the fungal isolate.

The culture with local isolates 3, 4, and 5 showed detectable contents of Aflatoxin B1, with concentrations 44.48, 29.58, and 20.62 ppm respectively. While low Aflatoxin B1 concentration has been showed with isolate 1(0.411 ppm), which wasn't to be enough detected by TLC. No Aflatoxin B1 was produced by isolate 2 (Table (1); Figure 2).

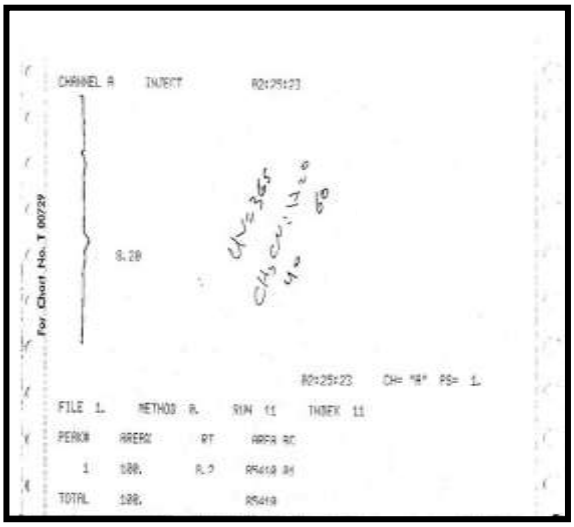
Table (1): Determination of Aflatoxin B1 produced by *A. flavus* isolates on coconut medium at 28±1° C after 10 days incubation using TLC and HPLC.

Fungal isolate	TLC	HPLC (ppm)
***Aspergillus flavus* No.1**	**-**	**0.411**
***A. flavus* No.2**	**-**	**0.0**
***A. flavus* No.3**	**+**	**44.48**
***A. flavus* No.4**	**+**	**29.58**
***A. flavus* No.5**	**+**	**20.62**

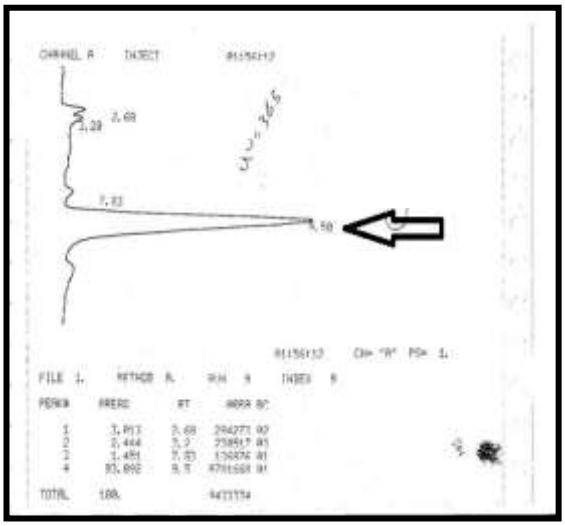
While thin –layer chromatography was frequently used in the past; HPLC has been used in recent years because of its ease of operation and better quantization. Most HPLC methods published to date have used reversed-phase HPLC on C18 bonded phases, where the Aflatoxins are separated by their hydrophobicity. Most published separations have been performed on 5 µm columns of 25-cm in length. The use of smaller particle size packing in shorter columns with faster separation times now in vogue. These columns show that the same separation can be achieved in less time than on the longer columns with similar resolution [21].



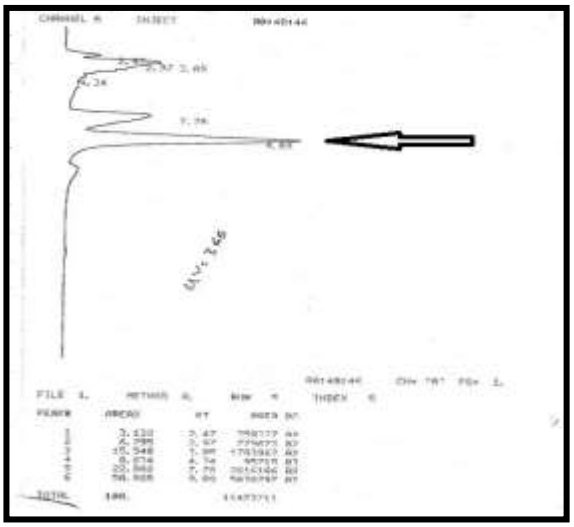
Isolate No. 1



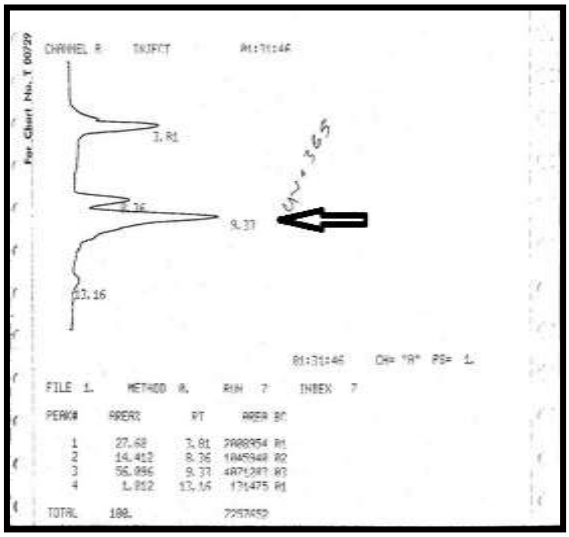
Isolate No. 2



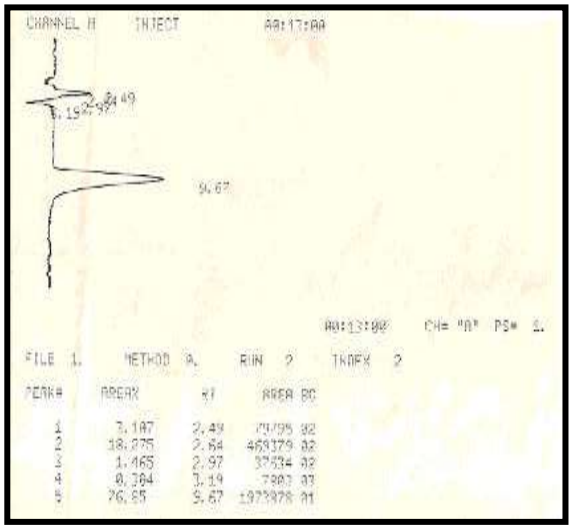
Isolate No. 3



Isolate No.4



Isolate No. 5



AFB1 standard (10ppm)

Fig. (2): Detection of Aflatoxin B1 produced from *Aspergillus flavus* isolates by HPLC

The DNA extracts were subjected to PCR analysis to confirm the possible presence of aflatoxigenic gene. As expected DNA from all aflatoxigenic Aspergilli produced clean bands upon amplification with *aflR1* set of specific primers. Mean while no band was detected on non aflatoxigenic fungi Figure (3).

Fig (3): Agarose gel analysis of PCR products from *A. flavus* Isolates using *aflR1* F and *aflR1* R Primers visualized under UV after staining with Ethidium bromide

1- Isolate number 1 (No Result)
2- Isolate number 2 (No Result)
3- Isolate number 3 (No Result)
4- Isolate number 4 (Product size 798bp)
5- Isolate number 5 (Product size 798bp)
L- DNA Ladder 100 bp (DNA molecular size marker

To confirm the specificity of PCR using another set of primers to detect the *aflR2* gene. All aflatoxogenic aspergilli showed positive results Figure (4) with expected size, Ca. 400 bp, of the fragment.

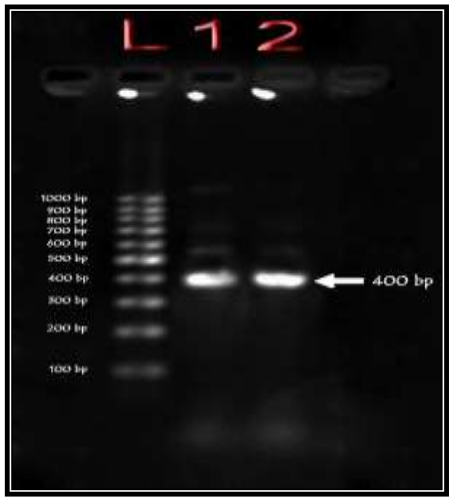


Fig (4): Agarose gel (1.5 %)analysis of Nested PCR products from *A. flavus* Isolates using *aflR2* F and *aflR2* R Primers visualized under UV after staining with Ethidium Bromide

1 – Isolate number 5 (Product size 400 bp)
2 – Isolate number 4 (Product size 400 bp)

Most of work in literature cited involve monomeric or multiplex PCR , which detect aflatoxiginic strains of *A. flavus*, *A. parasiticus* and *A. nomius*, but it does not always permit differentiation between aflatoxgenic and non aflatoxogenic strain . It is known

that food component can interfere with *Taq* polymerase giving false negative results [22].

It was able to detect Aflatoxin producing strains of *A. flavus* in contaminated figs by performing a nonnumeric PCR ,and by multiplex PCR after spiking and incubating the peanuts after 7days [18,23].

In the above mentioned reports, liquid nitrogen has been used for extraction of DNA and the detection of *Aspergillus* species had taken longer time (more than 24 hr). PCR protocols have been developed for pure culture systems, but detection of the same in food samples as limiting. Moreover, molds are found on dry food mostly as sexual spores or dried mycelia which contain only small amounts of DNA and are resistant to cellular disruption for DNA extraction [24].

The primers were specific for *aflR* gene fragment, the size of the amplicons corresponded to the expected size and no additional or non – specific bands were observed.

Nested PCR was used mainly to confirm the authenticity of the primery PCR. For *A. flavus*, the amplicons of 798 bp was reconfirmed by using it as a template in nested PCR. the nested PCR primers generated an expected size amplicon of 400 bp [20] Fig. (3,4) the DNA of isolate 4 and 5 were also subjected to PCR using *aflR* primers , but no amplicons were observed . Variation of DNA sequence can be detected by PCR – based methods therefore in order to discriminate between Aflatoxin producer strains and non – producers by using detailed comparison of PCR products of *aflR* gene fragment (Genbank accession number *A. flavus* AY197608) [25].

It is reported that genes involved in the Aflatoxin bio synthetic pathway may form the basis for an accurate, sensitive, and specific detection system, using PCR, for aflatoxigenic strains in grains and foods [24]. In this study, using primer designed to Aflatoxin regulatory pathway gene, *aflR*, the presence of aflatoxigenic fungi was easily detecting in compared to conventional plating techniques.

PCR in present study did not show any false priming results due to the presence of food components or any other contamination.

This technique is able to screen many, suspected samples in a time, resource saving manner in fine and expensive products of foods with highest possible accuracy.

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