Detection the Ability of *Aspergillus flavus* Isolated from Wheat Grains for Aflatoxin B1 Production using RT- PCR

التحري عن قدرة الرشاشيات الخضراء المعزولة من حبوب الحنطة على أنتاج السم الفطري افلاتوكسين B1 باستخدام تقنية RT- PCR

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Abstract

***Aspergillus flavus* is aflatoxinogenic and potential aflatoxins producers in agricultural commodities. The present study was conducted determine the ability of eleven strains of *A. flavus* isolated from Iraqi wheat grains *Triticum aestivum*. The isolates have been detected by molecular methods using Reverse Transcriptase RTPCR. In this study, RNA was extracted from *A. flavus*, cDNA synthesis and rapid assessment of eleven isolates of *A. flavus* was accomplished using primer pair for the aflatoxin regulatory gene *aflR* Reverse transcription-Polymerase chain reaction (RT–PCR).Positive amplification was achieved for all the isolates with a molecular weight 798 to *aflR1* and 400bp to *aflR2*. Also the result of the amplification showed there are no differences with the two molecular weight between the 11 isolated strains of *A. flavus* in their aflatoxin B1 production, but the first strain differed in their banding florescence as compared with others strains this reflect the genetic differences in aflatoxin B1 production between them.**

Keywords: AflatoxinsB1, *Aspergillus flavus*, RT–PCR

الملخص

الرشاشيات الخضراء *Aspergillus flavus* من الممرضات النباتية للمحاصيل الغذائية التي تسبب فساد وتلف المحاصيل عن طريق انتاج سموم الأفلاتوكسين. ركزت هذه الدراسة على تحديد قدرة إحدى عشر سلالة من الفطر *A. flavus* المعزولة من حبوب الحنطة الخبازية *Triticum aestivum* على أنتاج سموم الافلاتوكسين في العراق. وتم تشخيص هذه السلالات باستخدام تقنيه تفاعل البلمرة المتسلسل بوجود انزيم الاستنساخ الرجعي (RT-PCR). عزل الحامض النووي الرايبوزي RNA من عزلات الفطر*A. flavus* وتحويله الى الحامض النووي المكمل cDNA وتم التشخيص السريع للسلالات الفطرية باستخدام زوج من البرايمرات للجين المنظم لإنتاج السم الفطري AFLR وباستخدام تقنيه RT–PCR تحقق التضاعف لقطعة الحامض النووي cDNA بنتيجة ايجابية لجميع السلالات الفطرية بوزن جزيئي 798 زوج قاعدة للجين *AFLR1* و 400 زوج قاعدة للجين *AFLR2*. ونلاحظ أيضا إن جميع السلالات الفطرية قادرة على إنتاج السم الفطري افلاتوكسين بي 1 في كلا الأوزان الجزيئية (789 و 400 زوج قاعدة) ولكن في السلالة الأولى اختلفت في تألق الحزمة مقارنة بباقي السلالات وهذا يشير إلى الاختلافات الوراثية في إنتاج السم الفطري افلاتوكسين بي 1.

الكلمات المفتاحية: افلاتوكسين بي1, الرشاشيات الخضراء, RT–PCR

Introduction

Aflatoxins are Mycotoxins produced mainly by the aflatoxigenic fungi *Aspergillusflavus* [1]. *A. flavus* mainly infect wheat grains "*Triticumaestivum*", peanuts, tree nuts [2]. Foods can be contaminated by aflatoxigenic fungi, especially in tropical countries during preharvesting, processing, transportation and storage [3]. The risk of *A. flavus* comes from its ability to produce Aflatoxins spatially Aflatoxin B1. The *aflR* gene from *A.flavus* may be involved in the regulation of aflatoxin biosynthesis. The *aflR* gene product, AFLR, possesses a GAL4-type binuclear zinc finger DNA-binding domain [4].

Different methods are implemented to screen the ability of *A. flavus* strains for production of aflatoxin B1 some of these methods commonly using the culture of strains in suitable liquid or solid media. For this purpose many media are used: Yeast extract-sucrose (YES) [5]. Or Reddy medium, and natural media with wheat, rice, peanut, malt, date, palm kernel or coconut extracts [6].

Chemical methods like chromatographic methodsare used for detection of aflatoxin B1 in contaminated food and feed. However, these methods cannot detect aflatoxinogenic fungi in contaminated samples which contain undetectable amounts of aflatoxins [7]. Therefore we can use an excellent and direct methods like reverse transcription - polymerase chain reaction (RT-PCR) which has been devised as a method of RNA amplification and quantification after its conversion to cDNA. RT-PCR can be used

for cloning, cDNA library construction and probe synthesis [8]. cDNA serves as a template in PCR reaction [5].

The objective of the present study was to detect the production of Aflatoxin B1 by *A. flavus* strains using RT-PCR technique.

Materials and methods

***Aspergillus flavus* isolates**

A total of 11 *A. flavus* isolates isolated from wheat grains were collected from millers in Baghdad governorate. All Isolates were identified by morphological and microscopical features, and were sub cultured on Sabouraud Dextros Agar medium at 4^{o}C, for using them in aflatoxin B1 production.

Molecular study

RNA isolation and cDNA synthesis

After 7 days of *A. flavus* incubation on SDA medium, the entire mycelial colony was removed. The mycelia were quickly frozen in liquid nitrogen and stored at -80°C until extracted. Total RNA were isolated using Gene aid total RNA purification mini kit (Taiwan) according to the manufacturer's instructions. Isolated RNA was treated with RNase-free DNase I (Biobasic, Canada) for 20 min at 37°C, DNase was inactivated at 65°C for 10 min. The integrity of the RNA was verified after separation by electrophoresis on a 1.5% agarose gel containing 0.5% (v/v) ethidium bromide. First-strand cDNA was synthesized from 500ng of total RNA using Reverse Transcription system (Bioneer, Korea) with an oligo-dT_{15} primer. The reaction solution was used as Templates for reverse transcriptase polymerase chain reaction (RT-PCR).

Reverse transcription-Polymerase chain reaction (RT–PCR)

Primers were selected according to previously published studies [9]. These primers amplified the Aflatoxin B1 Regulatory Region (RR) for *A. flavus* isolates and B-actin (reference gene)cDNA were amplified using primers in Table (1)The sequences and name of PCR primers were listed at the following table: All primers were supplied by Alpha DNA Company, Canada.

Table (1): Primers used for amplification of *aflR1* and *aflR2* cDNA sequences.

Primer Gene	Primer	Sequence5'-3'	PCR product size (bp)	Genbank accession number	Reference
aflR1	Forward	AACCGCATCCACAATCTCAT	798	264763	[9]
	Reverse	AGTGCAGTTCGCTCAGAACA			
aflR2	Forward	GCACCCTGTCTTCCCTAACA	400	264764	[9]
	Reverse	ACGACCATGCTCAGCAAGTA			

Polymerase chain reaction was initiated with hot start method using the cDNA template on Lab net Thermo cycler (USA). 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 mMTris–acetate, 1 mM EDTA, pH 8.0) stained in 0.5 mg/ml ethidium bromide, *aflR1* cover the region from 540 to 1338 of aflatoxin regulatory gene with product size of 798 base pairs (bp) have been patented [9]. Nested PCR was carried out using the primer set *aflR2* 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 *aflR*2 [10].

Results and Discussion

All Isolates identifications were made on the basis of morphological and microscopical features as in figure (1), and were sub cultured on Sabouraud Dextros Agar medium at 4^{o}C, for using it in DNA extraction.

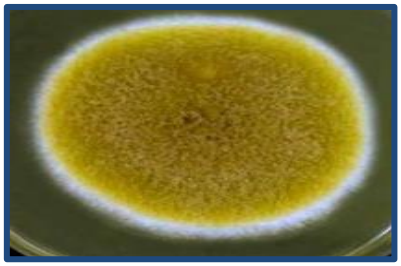
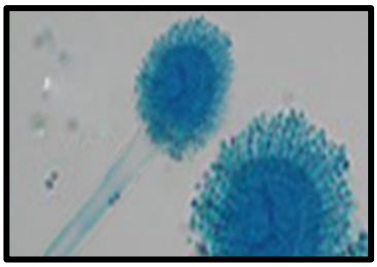


Fig.(1): Growth of *A. flavus* on culture media at 28°C for 7 days. Growth of *A. flavus* on PDA medium



Microscopic feature of *A. flavus* (staining with lactophenol cotton blue) showing conidial head (40 X)

The CDNA extracts were subjected to PCR analysis to confirm the possible presence of *aflatoxigenic* gene. The result of amplification indicated that all *A. flavus* examined produced clear bands upon amplification with *aflR1* set of specific primers figure (2).

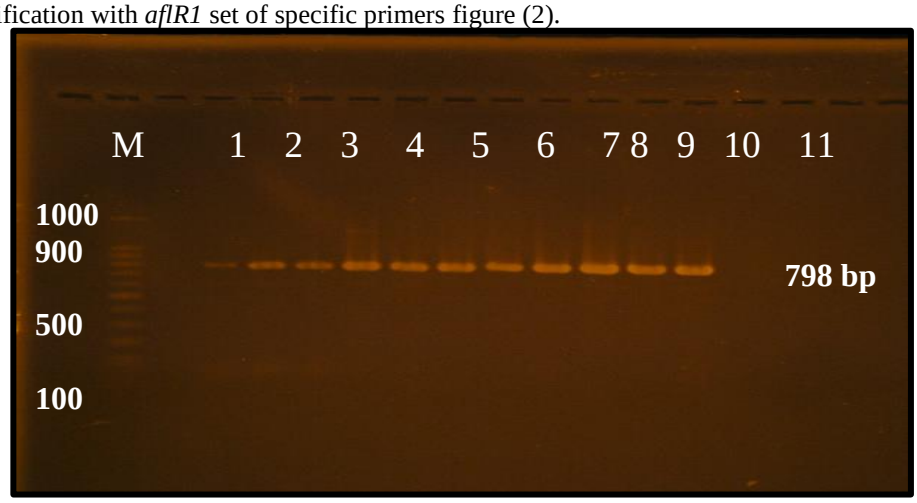


Fig.(2): Agarose gel (1.5 %) analysis of PCR products from *A. flavus* isolates using *aflR1* F and *aflR1* R Primers visualized under UV after staining with Ethidium bromide With a produced size 798bp. M- DNA Ladder 100 bp (DNA molecular size marker) -11 *A. flavus* isolates.

1 – 11 *A. flavus* isolates

To confirm the specificity of PCR results another set of primers were used to detect the aflR2 gene. All *A. flavus* isolates showed positive results Figure (3) with expected size, 400 bp, of the fragment as shown in figure (3).

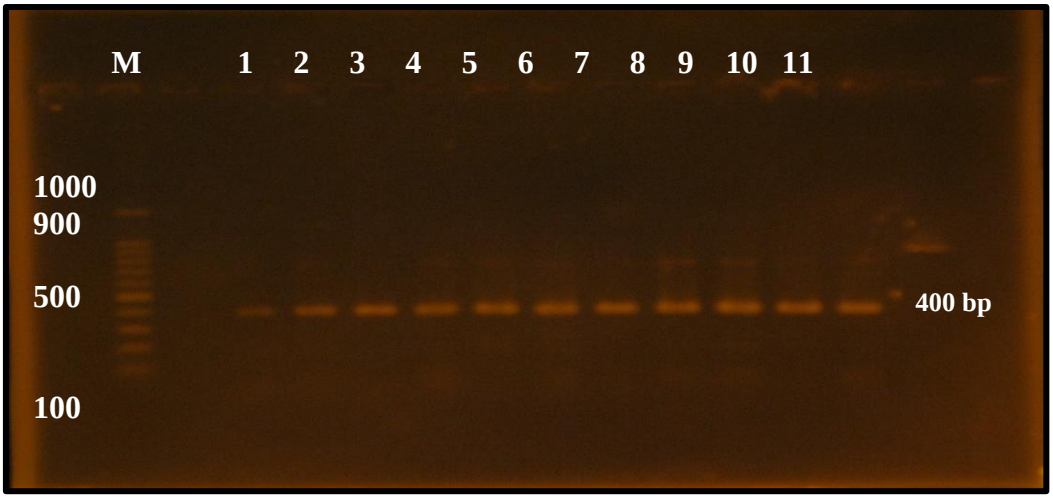


Fig.(3): Agarose gel (1.5 %) analysis of Nested PCR products from *A. flavus* Isolates using *aflR2* F and *aflR2* R Primers visualized under UV after staining with Ethidium Bromide with Product size 400 bp.

In this study, the RT-PCR reaction was targeted against aflatoxinB1 synthesis regulatory gene (*aflR*). The primary amplicon used as template for second PCR reaction was reacted with the *aflR2* set of primer provided for the nested amplification of *aflR2* gene present in aflatoxigenic isolates at molecular weight 400 bp.

Previous researches demonstrated that the *A flR* protein can bind the Promoter region of aflatoxin synthesis gene and activate *aflR* gene expression [11, 12]. So this explain the presence of *aflR* gene and un ability of some isolates to produce aflatoxin thus due to the failing in expression of *aflR* gene which can be related to the absence or un employed Aflr protein. The *aflR* gene has an auto regulation function, absence of the *aflR* gene or the presence of an abnormal *aflR* gene would be a strong indicator that isolates cannot produce aflatoxin [13].

Although there is no evidence of aflatoxin production by then on aflatoxin producing fungi like *A. oryzae*and *A. sojae*, some genes (*nor-1, ver 1, omt-A and aflR*) needed for aflatoxin biosynthesis are present, but not expressed, in these fungi [14, 15].

The optimal temperature for aflatoxin production is ranged between25˚C to 35˚C [16].Temperature affects aflatoxin production in *A. flavus* by altering the transcriptional profile, however the regulatory gene *aflR* was relatively constant at both temperature conditions, suggesting that the failure to produce aflatoxin at 37 ˚C is not due to the effect of temperature on the transcription of the pathway regulatory gene. *aflR*, possibly due to non-functionality at higher temperatures or alteration in *Aflr* [17]. When *A. flavus* was modified by adding extra – copies of two genes involved in aflatoxin production, amount of aflatoxin precursors was increased [18].

Conclusions

In conclusion, the results of molecular methods reported here for screening the ability of *A. flavus* to produce aflatoxin suggested that RT-PCR technique is the most suitable method to screen many. Suspected samples in a time are resource saving manner in fine and expensive products of foods with highest possible accuracy. Also from the detection results, the conclusion is there are no genetic differences between the 11 isolated strains in their alfatoxine B1 production except the first strain which differed in their banding fluorescence.

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