Detection of Aflatoxigenic and Non-Aflatoxigenic Isolates of *Aspergillus flavus* Isolated From Some Clinical and Environmental Sources by HPLC and PCR Techniques

الاعتماد على استشراب السائل عالي الأداء وتفاعل البلمرة المتسلسل في تحديد العزلات المنتجة للافلاتوكسين وغير المنتجة من *Aspergillus flavus* المعزولة من مصادر سريرية وبيئية

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Abstract

This study aimed to determine the role of polymerase chain reaction (PCR) and High-performance liquid chromatography (HPLC) technique in the discrimination between aflatoxigenic and non-aflatoxigenic isolates of *Aspergillus flavus*. The isolates were identified based on macroscopical and microscopical characteristics, and extracted aflatoxin was detected by HPLC technique. Furthermore, DNA was extracted from the all isolates and carried out by PCR to amplify target genes encoding for toxin production (*nor-1*, *ver-1* and *aflR*). The results showed that the genes (*aflR*, *nor*-1) were found in 11 (73%) of isolates, while the (*ver*-1) gene appeared in 10 (67%) of isolates. Both aflatoxigenic and non-aflatoxigenic isolates were also determined depending on the amplification of gene sites in the targeted DNA. HPLC technique has also used with high efficiency to ensure the aflatoxin-producing isolates and to evaluate the level of aflatoxin B1 production for 15 isolates of *A. flavus*. Ten isolates were able to produce aflatoxin with rates ranged from 0.78 to 45.03 ppm. PCR technique has proved high efficiency in the differentiation between aflatoxigenic and non-aflatoxigenic isolates of *A. flavus*. Moreover, aflatoxin production was directly associated with gene appearance and gene detection. Also, HPLC technique is a standard and superb technique in identifying and analyzing aflatoxin with high sensitivity and accuracy.

Keywords: Aflatoxin, *Aspergillus*, HPLC, PCR

الملخص

هدفت هذه الدراسة لتحديد دور تفاعل سلسلة البلمرة و تقنية الاستشراب السائلي عالي الأداء في التمييز بين العزلات المنتجة وغير المنتجة للافلاتوكسين من فطر *Aspergillus flavus*. شُخصت العزلات بالاعتماد على الخصائص المجهرية والمظهرية، و تم الكشف عن الأفلاتوكسين المستخلص بتقنية الاستشراب السائلي عالي الأداء. علاوةً على ذلك، تم استخلاص الحامض النووي لجميع العزلات و تم اجراء تفاعل البلمرة المتسلسل لتضخيم القطع المستهدفة (*nor-1*, *ver-1* and *aflR*) التي تشفر لإنتاج الافلاتوكسين باستخدام تفاعل البلمرة المتسلسل أظهرت النتائج أن الجينات *aflR* و*nor-1* ظهرت في 11 (73٪) من العزلات، في حين أن الجين *ver*-1 قد ظهر في 10 (67٪) من العزلات. كما تم تحديد كل من العزلات المنتجة وغير المنتجة للافلاتوكسين اعتمادا على تضخيم مواقع الجينات في الحمض النووي المستهدف. وقد استخدمت تقنية الاستشراب السائلي عالي الأداء بكفاءة للتحقق من العزلات المنتجة للأفلاتوكسين وتقييم مستوى إنتاج الافلاتوكسين B1 في 15 عزلة من الفطر *A. flavus*. عشر عزلات كانت لها القدرة على إنتاج الأفلاتوكسين بمعدلات تراوحت من 0.78 و 45.03 جزء من المليون. أثبتت تقنية تفاعل البلمرة المتسلسل كفاءة عالية في التمييز بين العزلات المنتجة وغير المنتجة للافلاتوكسين للفطر *A. flavus*. فضلاً عن ذلك، يرتبط إنتاج الأفلاتوكسين ارتباطا مباشرا مع تواجد الجينات والكشف عنها. كما تعتبر تقنية الاستشراب السائلي عالي الأداء تقنية قياسية وممتازة في تشخيص و تحليل الأفلاتوكسين بحساسية و دقةٍ عاليةٍ.

الكلمات الدالة: الافلاتوكسين، *Aspergillus* ، استشراب السائل عالي الأداء، تفاعل البلمرة المتسلسل

Introduction

Aflatoxins as effective carcinogenic toxins are secondary metabolites produced by several species of *Aspergillus*-like *Aspergillus flavus* [1]. These toxins are important because they can colonize a range of economically important crops such as maize, nuts, tree nuts and peanuts. It appears in deterioration, bad odor, and discoloration and it may contaminate them with aflatoxin. Featuring these toxins have a high acute toxicity [2]. There are many factors affecting aflatoxin production during the storage period such as temperature, moisture, availability of oxygen, and fat content. There are four types of aflatoxins (B1, B2, G1, and G2) while

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aflatoxin B1 is the toxic type which is more dangerous [3].

The cluster gene pathway for aflatoxin may allow all of the pathway genes to be expressed rapidly upon the appearance of secondary metabolites. Therefore, aflatoxins start to accumulate rapidly after 18 to 20 h of mycelial growth [4]. A distinction between aflatoxigenic and non-aflatoxigenic isolates of *Aspergillus* section Flavi has been a matter of controversy [5]. Conventional methods which used for detection of aflatoxins are microbiological identify, high-performance liquid chromatography (HPLC), thin layer chromatography (TLC) or enzyme-linked immunosorbent assay (ELISA). These procedures are time-consuming though they are considered reliable techniques in detection of aflatoxins [6]. However, polymerase chain reaction (PCR) is easy to be used, rapid and accurate [7] .

Because of the spread contamination of food products with *A.flavus* and the difficulty of access to a cut-off diagnostic tool for differentiation of aflatoxin producing isolates in addition to molecular screening and detection for aflatoxin encoding genes (*ver-1*, *nor-1*, and *aflR*). Thus, the aims of our study are to (1) determinate the capability of *A. flavus* isolated from clinical and environmental sources for production of aflatoxin B1, (2) differentiate between aflatoxigenic and non-aflatoxigenic isolates of the above fungus depending on PCR in addition to molecular detection for aflatoxin encoding genes (*ver-1*, *nor-1* and *aflR*), and (3) investigate the role of HPLC in analyzing and identifying aflatoxin B1 (AFB1) level produced from *A. favus* obtained from well extracted samples.

Materials and methods

Identification of fungal isolates

Fifteen *A. flavus* isolates were obtained from two laboratories; five (33%) clinical isolates were obtained from the laboratory of College of Veterinary Medicine-University of Baghdad while 10.0 (67%) environmental isolates were obtained from laboratories of the Environment and Water Department, Ministry of Science and Technology. These isolates were examined directly under the microscope using 10% KOH and culturing on Sabauroud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA) [8]. *A. flavus* isolates were cultured on SDA by the addition of 0.05 mg/ml Chloramphenicol to inhibit the growth of bacteria, then incubated at 28°C and 37°C and examined for seven days according to Duniere *et al*,. (2017). The isolates were identified depending on the species level based on macroscopical and microscopical characteristics using SDA [8] and scotch tape preparation [10]. All fungal isolates which identified as *A. flavus* were cultured and maintained in PDA supplemented with chloramphenicol and incubated for five days at 28±2°C [11].

SDA slants were prepared and sterilized according to Lahouar *et al*., (2016). After that, chloramphenicol of 50 mg/l was added. A loop was used for taking spores to implant in test tubes then incubated 28 C for 7 days. After incubation of 10 ml of sterile distilled water in culture tubes with *A.flavus* [13], the spores were harvested by sterile loop and suspension of spores was prepared in the tubes. After transferring and dilution of spore suspension, the spores were accounted in five µL by using a hemocytometer [14].

According to Lai *et al*., (2015), each flask was inoculated with million spores and incubated at 28 ± 1 °C in the dark for 21 days and shaken once or twice daily for three days to aid in the even distribution of the inocula. After fermentation, the flasks were placed briefly in the oven at 60 C for three hours to destroy the fungus.

Extraction and detection of Aflatoxin B1 by HPLC

Aflatoxin has been extracted according to Association Official Analytical Chemists 2000, procedure with few modifications as follows: 25 grams of rice powder who previously contaminated with A.flavus spores was added to 25 ml of chloroform and water (1:1 v/v). The contents of the flasks were shaken for 60 minutes. The crude extracts were filtered through gauze, and then through Whatman filter paper (No. 1). 25 ml of filtrate was mixed with 25 ml methanol 90% and hexane at (1:1 v/v) in a separate funnel for 10 minutes. The upper layer was discharged and the methanol lower layer was evaporated to near dryness and then 25 ml chloroform/water (1:1 v/v) was added to the extract to another separate funnel. The funnel was shaken thoroughly for about 1 minute, then the lower layer chloroform passed through filter paper contained 10 g of anhydrous sodium sulfate. The last filtrate was evaporated to near dryness and the residue was stored in small dark vials at 5°C for chemical analysis. Aflatoxin B1 has been detected by high-performance liquid chromatography (HPLC) with the following conditions: column: 250x4.6mm, particle size 5 µm, ODS (C18), mobile phase acetonitrile: water 40:60 v/v, flow rate 1ml/ minute., detector UV-365nm and compared to standard aflatoxin B1 (Sigma) as control, as in Figure (2).

Extraction of total DNA from mycelia

Total DNA was extracted from 0.5 g (wet weight) of mycelia after harvested, fungi growth was on enrichment culture (SDA) medium for 5 days. The mycelia/spores were frozen in liquid nitrogen which freezes the wall of the mycelia. After that, they transferred into a mortar to be milled in mycelia and crushed well. The temperature of liquid nitrogen was -196 ºC, and grinding continued for a 1 minute so that the fungus becomes a powder. A grounded sample was transferred into clean micro tubes with the capacity of 1.5 ml [17]. Twenty µl of Proteinase K, cell lysis buffer was added to the samples and mixed thoroughly by vortex, and incubated at 60°C for 15 minutes to ensure the samples lysate and mix by vortex. Then, 100 µl of the protein precipitation solution was added to the mixture, mixed by vortex immediately for 10 seconds. The mixture was incubated on ice for 5 minutes. The 1.5ml supernatant was transferred to new clean tubes containing room temperature Isopropanol. An aliquot of 300 µl Isopropanol was added and mixed thoroughly by vortexing for 20 times. The mixture was mixed by inversion and centrifuge at 15,000×g for 2 minutes. Seventy % of room temperature ethanol was poured to the supernatant, and the mixture was centrifuged at 15,000×g for 2 minutes. DNA Rehydration Solution was added. 1.5 µl of RNase was added and incubated at 37 ºC for 15 minutes and Rehydrated at 65 ºC for 1 hour or overnight at 4 ºC. The purity and integrity of the isolated DNA were determined by NanoDrop Microvolume Spectrophotometers and agarose gel electrophoresis 1%.The genomic isolation kit was provided by Promega kit, USA

Target genes and PCR reagents

Total DNA was used for detection of genes which encode to aflatoxin produce in *A.flavus* isolates, polymerase chain reaction (PCR) was conducted for amplifying three genes i.e. (*ver-1*, *nor-1* and *aflR*). These genes and specific primer sequences have been selected from the already reported data [18]. The primer sequences, the name of genes and their product size are shown in Table 1. All PCR reagents were provided and synthesized by (Bioneer-Korea), Primers were scientifically designed thematically and all PCR attempts were carried out in PCR Thermal Cycler (Applied Biosystems).

Table (1): Primers used and their sequences

No.	Primers Name	Sequence(´5-´3)	Product size	References
1	*nor 1 (aflD)*	nor1 ACCGCTACGCCGGCACTCTCGGCAC nor2 TTGGCCGCCAGCTTCGACACTCCG	400 bp	Rashid *et al.*, (2008)
2	*ver 1 (aflM)*	ver1 GCCGCAGGCCGCGGAGAAAGTGGT ver2 GGGGATATACTCCCGCGACACAGCC	600 bp	Rashid *et al.*, (2008)
3	*aflR*	aflR1 TATCTCCCCCCGGGCATCTCCCGG aflR2 CCGTCAGACAGCCACTGGACACGG	1000 bp	Rashid *et al.*, (2008)

Optimization of PCR

PCR reaction kit (premix) was selected from (Bioneer, Korea). The PCR reaction was carried out in 25 µl solution containing (5 µl) premix (Taq DNA polymerase, 250 µM (each) dATP, dGTP, dCTP, dTTP and 1.5 Mm MgCl2, reaction buffer (PH 9) and loading dye buffer (yellow and blue dye), 2 µl each of amplification primers, 4 µl target DNA, 12 µl free nuclease water. Thermal cycle conditions were carried for these three genes Table (2). PCR amplified products were checked on 1% gel by gel electrophoresis and visualized under Gel documentation system [18].

Table (2): The PCR program which was used in the amplification of the targets DNA for*: aflR, nor1* and ver 1 [18]

Steps	Temperature (°C)	Time	
Initial denaturation	95	4 minutes	
Denaturation	95	1 minute	30 cycles
Annealing	58 – 62	1 minute	
Extension	72	30 second	
Final extension	72	10 minutes	

The Statistical Analysis System-SAS (2012) program was used to reveal the effect of different factors in study parameters. Chi-square test was used to compare between percentages and the least significant difference (LSD) between means [19].

Results and discussion

Isolates were cultured on two different types of media (SDA and PDA) for isolates conservation and all showed good growth. For more experiment, the *A.flavus* cultures were potato dextrose agar (PDA). To increase DNA amount and obtain pure colonies from *A.flavus*, enrichment method was adopted in which fungus from environmental and clinical sources was incubated on enrichment medium as explained by Quinn *et al.*, (2013) was used which showed the remarkable growth of fungal mycelia in the form of powdery colonies on SDA media. The enrichment method was followed by DNA extraction from these fungal colonies, which was now readily extractable with high purity, for detection aflatoxigenic fungi by PCR .

Grains of rice were chosen as a culture media to diagnose aflatoxigenic and non-aflatoxigenic isolates growth by using HPLC approaches. The uninfected grains of rice were first artificially inoculated a definite number of spores (million spores) to examine whether the rice got affected by these fifteen *A. flavus* isolates.

All isolates on rice got infected effectively Figure (1). Only ten isolates were able to show aflatoxin product on rice which was confirmed by PCR in detection of genes (*aflR*, *nor-1* and *ver-1*) that encoded aflatoxin. Some of the results are shown in Table (3).

Fig. (1): Fluffy mycelia growth of *A. flavus* in ME broth after incubation of 7 days

In the current study, we have found a fast, sensitive and specific technique to be used in the detection of aflatoxigenic and non-aflatoxigenic isolates taken from clinical and environmental sources. The strategy allows for processing a large number of samples in obtain results in the short time compared with conventional methods [21].

The results showed that out of 15 isolates, 11 (73%) were positive for the *aflR* gene and *nor-1* gene, while 10 (67%) isolates were positive for *ver*-1 gene. Also, amplification of *nor*-1 gene targeted by PCR and electrophoresis by agarose gel electrophoresis revealed that out of 15 isolates of *A. flavus*, 11 (73%) isolates were positive for *nor*-1 gene, while four (27%) isolates were negative as reflected in the following figure (2).

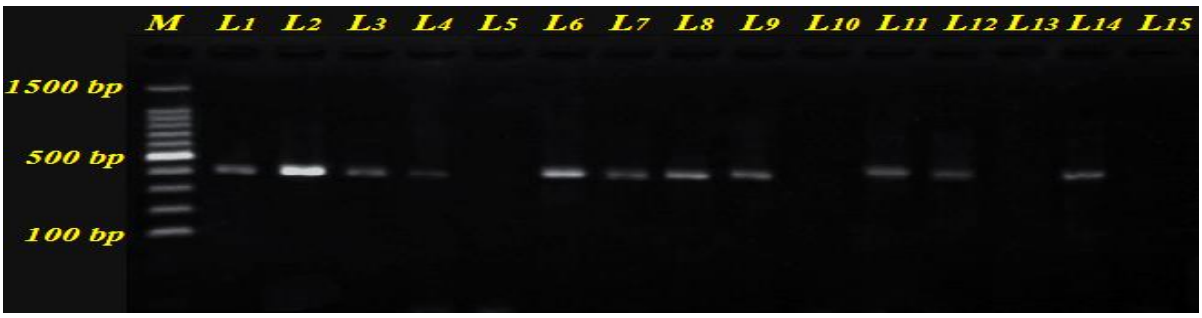

Fig. (2): PCR product with *nor*-1 primer (specific for *nor*-1) on 1 % agarose gel electrophoresis with ethidium bromide, M: 100 bp DNA ladder. Lanes: AFl1, AFl2, AFl3, AFl4, AFl5, AFl6, AFl7, AFl8, AFl9, AFl10, AFl11, AFl12, AFl13, AFl14, AFl15.

Aflatoxigenic isolates were the prevailing species of among all isolates [6]. This study was conducted because *A. flavus* was observed as the most commonly isolated from environmental and clinical sources [22]. Notwithstanding of this, no attempt was performed to identify aflatoxin producing species with using fast molecular techniques in clinical and environmental isolates [23]. The gene *nor*-1 plays a great role in the early conversion of the norsolorinic acid to averantin (in the middle of AF biosynthetic pathway), while other genes are involved in converting sterigmatocystin to AF in the last step of the aflatoxin pathway [24].

The absence of the genes encoding for aflatoxin production in *A. flavus* genome indicates the inability of this isolate to produce aflatoxin [25]. Moreover the non-aflatoxigenic isolates such as isolate (AFl1) lacking *ver*-1 gene. Whereas showed the presence of *aflR* and *nor*-1 genes, PCR indicated that the gene does not exist in this isolate Table (3). This may be due to the fact that the ver-1 gene encodes an enzyme a ketoreductase which is required for the conversion of versicolorin A to desmethyl sterigmatocystin (DMST) in the aflatoxin biosynthetic pathway [26]. The presence of the *ver*-1 gene, which is in charge of the production of aflatoxins, was shown in 10 isolates of the *A. flavus* from a total of 15 isolates as in figure (3). Where it was noted that the site of the gene appeared in the sequence (600 bp) when the primer *ver*-1 was used.

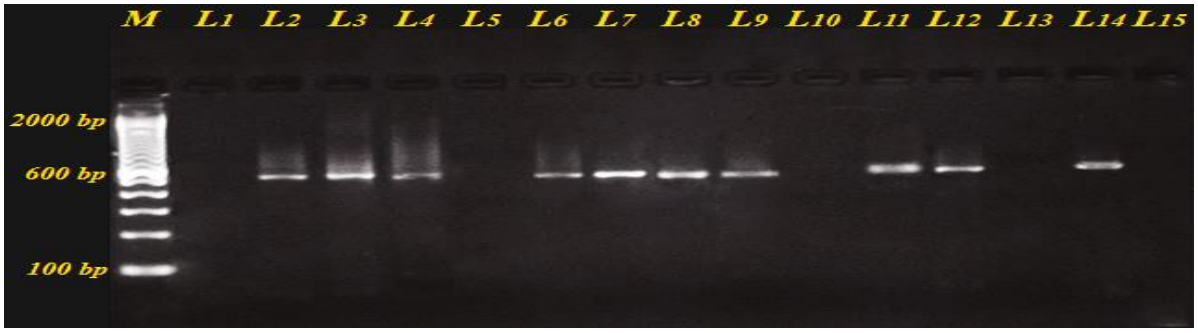

Fig. (3): PCR product with *ver-1* primer (*ver*-1 gene) on 1.5% agarose gel electrophoresis with ethidium bromide, M: 100 bp DNA ladder. Lanes: AFl1, AFl2, AFl3, AFl4, AFl5, AFl6, AFl7, AFl8, AFl9, AFl10, AFl11, AFl12, AFl13, AFl14, AFl15.

This result corresponds with the findings of Hamed *et al*., (2016). The findings of these studies were in agreement with the results of previous studies on the chain of DNA sequence that belongs to the gene ver-1 appropriate design significant primer to diagnose fungi producing aflatoxins [27]

The *aflR* primer pairs were selected to detect the aflatoxigenic isolates of *A. flavus* in the PCR technique as a marker because this gene has importance in the biosynthesis of aflatoxin, amplification of *aflR* gene targeted by PCR and electrophoresis. The results revealed that the ability of 10 isolates out of 15 isolates to produce AF through the gene *aflR* which is responsible for regulating the AF biosynthetic process. Figure (4) showed the PCR products (1000 pb).

Although these specific primers to amplify the expected bands from the isolates produced for aflatoxin, great variability was usually found in their aflatoxigenic capacity tested by the HPLC method which showed that it was not a sufficient marker for differentiation between aflatoxigenic and some non-aflatoxigenic isolates. The lack of aflatoxin production could also be due to simple mutations (substitution of some bases) which lead to the formation of nonfunctional products. Lack of aflatoxin production apparently can also be related to the incomplete pattern obtained in PCR [28,29].

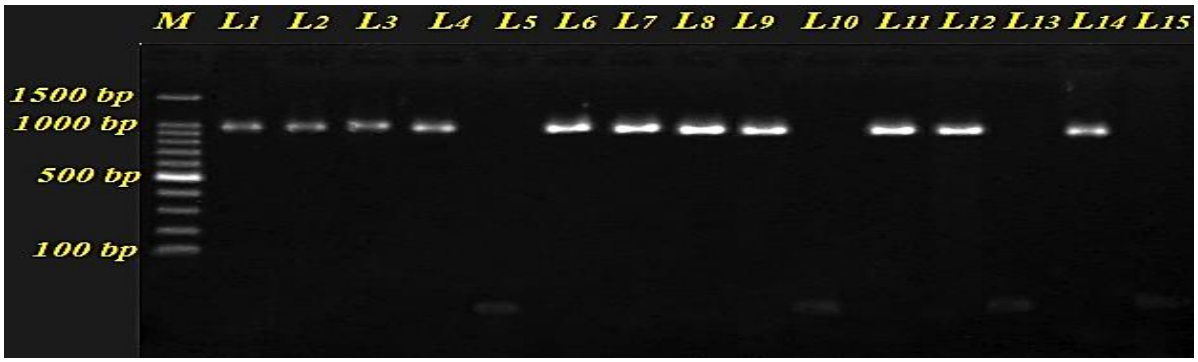

Fig. (4): PCR product with an *aflR* primer on 1 % agarose gel electrophoresis with ethidum bromide, M: 100 bp DNA ladder. Lanes: AFl1, AFl2, AFl3, AFl4, AFl5, AFl6, AFl7, AFl8, AFl9, AFl10, AFl11, AFl12, AFl13, AFl14, AFl15.

The biosynthetic pathway includes many enzymatic steps including aflatoxins as the end product. Notwithstanding earlier work, there is yet to be detected a definite marker for producing aflatoxins, independent from their measurement [30]. Several studies have reported the use of PCR technique as a sensitive and rapid method for detection and determination of aflatoxin production [31] and identifying aflatoxigenic isolate from non-aflatoxigenic isolate [18]. PCR analysis was capable to amplify regulatory gene *aflR* and two structural genes i.e. *nor*-1 and *ver*-1 and by using specifically designed primers. The *aflR* is the positive regulatory gene which acts required for transcription activation of most of a structural gene [26].

In this study, 10 isolates out of 15 were aflatoxigenic when examined by PCR analysis. As it was noted, nor-1 gene was amplified in most of the isolates as it was a structural gene required in the step of initial for biosynthesis pathway of aflatoxin. Three primers were thoroughly selected to be very specific for these three genes perceived to be necessary for aflatoxin biosynthesis. This primer pair yielded a single DNA fragment of the predicted size of 400, 600 and 1000 bp for (*nor-1*, *ver-1* and *aflR*) respectively. Interestingly, all the ten isolates were able to confirm the aflatoxin production by using HPLC. This technique have been used as a strategy to confirm aflatoxin production from isolates which show amplification of three genes (*nor-1*, *ver-1* and *aflR*), but with the unsteady level of success which can be accomplished by many attempts by using PCR [32].

HPLC has been recognized as a standard and superior method in the identification and analyzing aflatoxin with higher sensitivity and accuracy in comparison with other methods [6]. The isolates of the study were examined by using HPLC techniques with the ultraviolet radiation detector at a wavelength of 365 nm. The results showed the ability of most isolates of A. flavus to produce AFB1, as it gained the value of the retention time (RT) for each of the inoculated samples of extract and the sample of the standard solution B1 (7.30) minutes figure (5).

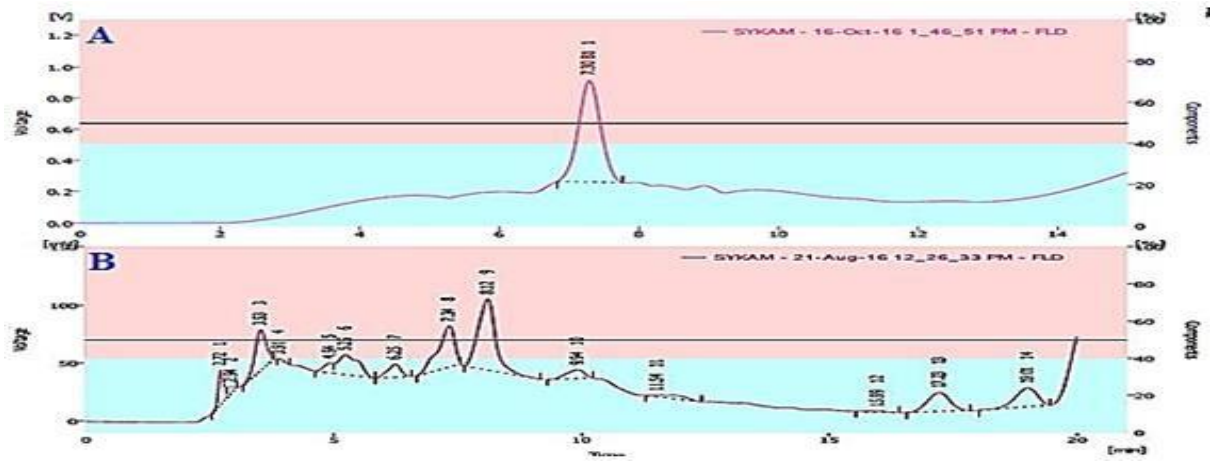

Fig. (5): Detection of AFB1 extracted from *Aspergillus flavus* by HPLC, the Standard chromatogram of AF (A): (Total Run Time: 14 minutes Retention time for AFB1: 7.30 minutes), (B): Chromatogram of various AF in *A. flavus*

The study results obtained from HPLC revealed that non-aflatoxigenic isolates were less detected than aflatoxigenic isolates. Also, aflatoxin production is directly associated with the appearance of genes (*ver-1*, *nor-1*, and *aflR*) [23]. The analysis of HPLC results indicated that *A. flavus* in peanut produced the highest concentration of aflatoxin B1at a rate of 45.03 ppm while the rate of *A. flavus* in crude oil was 0.78 ppm (least rates of aflatoxin B1 Table (3).

Aflatoxigenic activity in the environmental and clinical isolates was tested in which 10 samples out of 15 gave the positive results for aflatoxin contamination. Non-aflatoxigenic isolates showed different patterns by noting no amplification. All the isolates having less than three target DNA fragments were deemed to be non-aflatoxigenic.

Table (3): Distribution the results of HPLC and gene detection by PCR according to study fungal isolates from clinical and environmental origin.

Isolate No.	Isolate origin	Co. AFB1 in HPLC (ppm)	Aflatoxin genes		
			aflR	*nor-1*	*ver-1*
AFL1	Fish	0.00 ± 0.00	+	+	-
AFL2	Lung of animal	11.66 ± 0.75	+	+	+
AFL3	Spices	12.55 ± 0.87	+	+	+
AFL4	Rice	8.35 ± 0.63	+	+	+
AFL5	Animal waste	0.00 ± 0.00	-	-	-
AFL6	Corn grain	33.86 ± 2.04	+	+	+
AFL7	Crude oil	0.78 ± 0.01	+	+	+
AFL8	Barley grain	6.24 ± 0.58	+	+	+
AFL9	Wheat	29.89 ± 1.93	+	+	+
AFL10	Bovine Milk	0.00 ± 0.00	-	-	-
AFL11	Fruits	17.39 ± 1.26	+	+	+
AFL12	Nuts	12.66 ± 0.73	+	+	+
AFL13	Poultry lung	0.00 ± 0.00	-	-	-
AFL14	Peanut seeds	45.03 ± 2.59	+	+	+
AFL15	Vegetables	0.00 ± 0.00	-	-	-
	LSD	6.593 *			

[a]means presence of PCR amplification signal, [b]means absence of PCR amplification signal

HPLC study results agreed completely with those obtained by molecular technique, PCR. There was a relationship between the result of the study of PCR and HPLC technique. Thakar *et al*., (2017) stated that the lack of aflatoxin production can be a result of the substitution of some nitrogen bases. Also, Ibrahim *et al*., (2016) concluded that various physiologic conditions can be effective in aflatoxin biosynthesis. In this study, it was observed PCR may be considered as a screening test for initial isolation regarding high sensitivity and speed (100%). The positive samples need more investigation by an efficient technique such as HPLC.

In this study, it is concluded that PCR technique is proved to be the accurate and rapid detection system in discrimination between aflatoxigenic and non-aflatoxigenic isolates of *A. flavus* on the basis of amplification of the target DNA fragments that are involved in aflatoxin production. Further, aflatoxin production is directly associated with an appearance of genes (*ver-1*, *nor-1*, and *aflR*). The present study demonstrated that all the ten isolates of *A. flavus* which showed amplification in three of genes and were able to confirm the aflatoxin production when they are diagnosed by using HPLC, this technique is standard and superior in identifying and analyzing aflatoxin with high sensitivity and accuracy.

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