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

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Abstract
Aspergillus flavus is aflatoxigenic 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 RT-PCR. 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

Introduction
Aflatoxins are Mycotoxins produced mainly by the aflatoxigenic fungi Aspergillus flavus [1]. A. flavus mainly infect wheat grains “Triticum aestivum”, 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 methods are used for detection of aflatoxin B1 in contaminated food and feed. However, these methods cannot detect aflatoxigenic 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 \textit{A. flavus} strains using RT-PCR technique.

\textbf{Materials and methods}

\textit{Aspergillus flavus} \textit{isolates}

A total of 11 \textit{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\textdegree C, for using them in aflatoxin B1 production.

\textbf{Molecular study}

\textbf{RNA isolation and cDNA synthesis}

After 7 days of \textit{A. flavus} incubation on SDA medium, the entire mycelial colony was removed. The mycelia were quickly frozen in liquid nitrogen and stored at -80\textdegree 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\textdegree C, DNase was inactivated at 65\textdegree 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-d\textsc{T}15 primer. The reaction solution was used as Templates for reverse transcriptase polymerase chain reaction (RT-PCR).

\textbf{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 \textit{A. flavus} isolates and \textit{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.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Primer} & \textbf{Gene} & \textbf{Primer} & \textbf{Sequence5'-3'} & \textbf{PCR product size (bp)} & \textbf{Genbank accession number} & \textbf{Reference} \\
\hline
Forward & aflR1 & AACCGCATCCACAATCTCAT & 798 & 264763 & [9] \\
Reverse & & AGTGCAGTTCGCTCAGAACA & & & \\
Forward & aflR2 & GCACCCGTGTCTCCCTCAA & 400 & 264764 & [9] \\
Reverse & & ACGACCATGCTCAGCAAGTA & & & \\
\hline
\end{tabular}
\caption{Primers used for amplification of aflR1 and aflR2 cDNA sequences.}
\end{table}

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 95\textdegree C for 10min. A total of 30 PCR cycles, each cycle at 0.3min at 94\textdegree C for denaturation, 0.45 min at 55\textdegree C for annealing, 1.15 min at 72\textdegree C for extension and a 10min final extension at 72\textdegree C. 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, \textit{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 aflR2 [10].

\textbf{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\textdegree C, for using it in DNA extraction.
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).

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).
In this study, the RT-PCR reaction was targeted against aflatoxin B1 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 aflR 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 between 25°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.

References


