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PCR-Based Detection of Aflatoxogenic Strains of Aspergillus flavus Isolated from Poultry Feed

R. N. Alkhursan1, B. A. Abbas2*and M. H. Khudor2

1Department of Biology, College of Science, University of Basrah, Basrah, Iraq
2Department of Microbiology, College of Veterinary Medicine, University of Basrah, Basrah, Iraq

Email: *basil.abbas@uobasrah.edu.iq*

Abstract. Twenty five isolates of A. flavus were detected by UV light (365nm) and ammonia vapor procedures. Aflatoxigenic A. flavus on Coconut Agar Medium (CAM) colored the reverse of glass petri dish with blue –green under UV light and produce a pink to red color by exposure to ammonia vapor. The detection by fluorescent blue revealed that 13 (52%) of isolates were aflatoxigenic by produce fluorescent color under UV (365nm) light , and also 13 (52%) of isolates were aflatoxigenic (positive) by ammonia vapor test. The molecular assessment was done on 25 isolates of A. flavus by using aflR gene primers. Five isolates of aflatoxigenic A. flavus positive identified isolates by PCR were randomly selected to sequence and analyze by basic local alignment search tool analysis (BLAST) to confirm the aflatoxigenic strains. Five isolates were positive and confirmed approximately compatible (99%-100%) homology with other A. flavus strains on NCBI.

1. Introduction

Molds can grow and produce mycotoxins in pre-harvest or during storage, transport, processing or feeding and these periods; humidity and temperature play an important role in the fungi growth and mycotoxins contamination [1]. In wet feeds, higher moisture levels allow mold growth if oxygen is available [2]. Feeds containing more than 12-15% moisture suitable to grow fungi and because most molds are aerobic, high moisture concentrations can eliminate air and prevent mold growth [3]. Two methods were used for determination of mycotoxins in food including conventional methods and molecular methods such as PCR and pulsed-field gel electrophoresis [4].
Mycotoxins are low molecular weight [5]. Mycotoxins resist decomposition or being broken down in digestion and thermally stable , so they remain in the food chain even after heat treatment, such as cooking and freezing [6; 7; 8]. Polymerase chain reaction (PCR) is a sensitive and specific technique to detect toxigenic molds early to control or reduce mold mass [9]. Unique DNA sequences must be selected as primer binding sites. Because of its sensitivity and specificity, PCR is an attractive method for the detection of fungi. [10]. DNA sequences that are polymorphic between fungal species, such as internal transcribed spacer (ITS) regions, are good candidates for the detection of a species to the exclusion of all other species [11]. This method with specific fungal primers is a powerful technique not only in diagnostics but also in ecological studies for screening fungi in natural environments, such as water, soil, plant or clinical samples. A biomolecular technique (PCR) was applied by using a set of primers of aflatoxigenic genes (aflR, aflD, aflM and aflP) to distinguish between aflatoxigenic strains and non-aflatoxigenic strain of A. flavus and A. parasiticus contaminating food and feed [12].

2. Materials and Methods

2.1. Detection tools of aflatoxigenic A.flavus

These tests were carried out on 25 isolates of A. flavus to detect aflatoxigenic or nonaflatoxigenic isolates by UV light, ammonia vapor and molecular detection by PCR.

2.2. Coconut based medium detection

A preliminary screen for aflatoxin producer A. flavus was done based on blue to blue – green fluorescence emission by light of UV irritation at 365 nm when the isolate was grown on CAM ,this agar is inductive of aflatoxin production in glass Petri dishes [13, 14, 15]. Use 5 mm diameter sterile cork borer to make a hole in the center of CAM medium in Petri dish. A mass of conidia of isolates was inoculated of by cork borer to hole at the centric point of CAM in glass Petri dish, then they were incubated at 28 °C for 7 days. The isolates of aflatoxigenic A. flavus appeared blue to blue-green fluorescence under UV light with long wavelength of 365 nm, while the isolates of non aflatoxigenic A. flavus remain colorless. Isolates of A. niger under the same conditions, was used as nonaflatoxigenic control [15, 16].

2.3. Ammonia vapor detection

The isolates of A. flavus were inoculated on CAM by cork borer (5mm) diameter in the center of plate and incubated in the dark at 28 °C. for 7 days. The dish was upended, then 1 or 2 drops of ammonium hydroxide solution (concentrated) are put on the lid inside of the petri dish. The Petri dish inverted over the lid containing the ammonium hydroxide. The colonies of aflatoxin-producer A. flavus rapidly turn reddish pink after the bottom of the culture. No color change occurs in colonies of non aflatoxins producer A. flavus [17] . A control as was mentioned in the previous test was prepared.

Molecular assay.

2*.4. DNA extraction and PCR*

DNA extracted from freshly growing cultures of A. flavus mycelia harvested on potatoes dextrose agar medium. Grinding the mycelium into a fine powder by liquid nitrogen using a pre-cooled pestle, then transferred in an Eppendorf tube. The genomic DNA was extracted by using a fungal genomic DNA extraction mini-preps kit(Bio Basic / Canada).

PCR was used to amplified aflR fragments of aflatoxigenic A. flavus genomic DNA. This assay is designed to include the examination of A. flavus isolates by extraction of their DNA and using the PCR technique. The following primers (F: aflR-1: ´5 -AACCGCATCCACAATCTCAT-3´ and R: aflR-2: ´5-AGTGCAGTTCGCTCAGAACA-3´) were used leading to a product with molecular weight 798bp [18].

2.5. Sequencing of PCR products for aflR gene

Five PCR products of aflatoxigenic A.flavus that identified by PCR were randomly selected , sequenced at Macrogen company in South Korea . The sequenced aflR products were analyzed homology with standard sequences of aflR gene deposited to NCBI gene bank using BLAST analysis software at Http://blast.ncbi.nlm.nih.gov/Blast.cgi (19).

3. Results and Discussion

Twenty five isolates of A. flavus were selected to test by coconut based medium and ammonia vapor in to determine either aflatixigenic or non aflatoxigenic ability of these isolates. These tests revealed that 13 (52%) of isolates were positive aflatoxigenic and 12(48%) of isolates were negative or nonaflatoxigenic (Table 1).

3.1. Molecular detection

Twenty five isolates of A. flavus were tested in order to determine either aflatixigenic or non aflatoxigenic isolates. This test confirmed that 17 (68%) were aflatoxigenic isolates(positive) by PCR and 8(32%) were nonaflatoxigenic (figure 1).

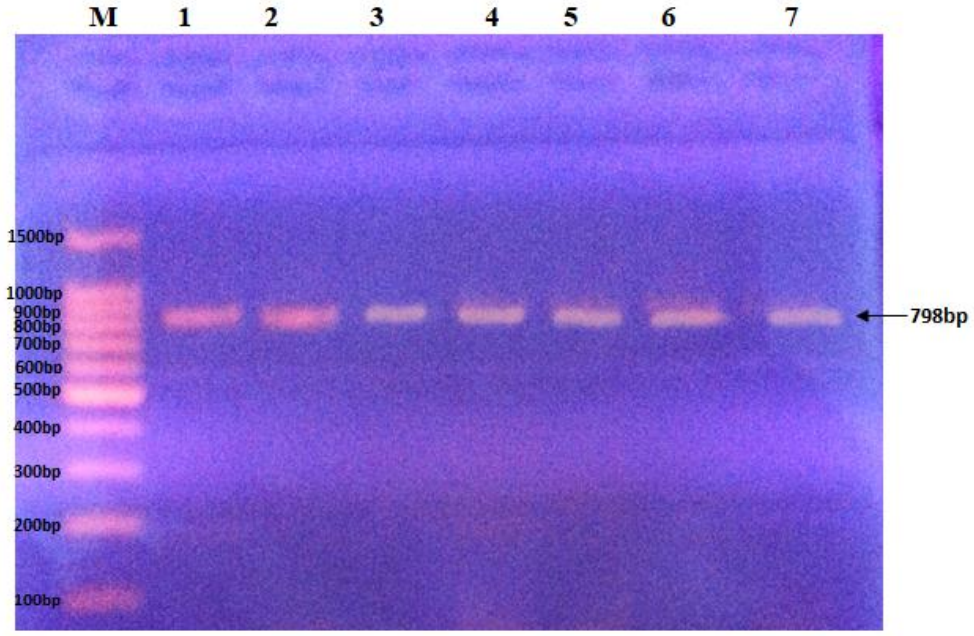

Figure 1: PCR products obtained through agarose gel electrophoresis from DNA of A.flavus isolates showing amplicons for aflR primer. Lanes: M- 100bp ladder, Lanes 1–7: A. flavus (aflatoxin producer) in corresponding to 798 bp.

There was the difference in the number of a positive result in the identification of isolates of aflatoxigenic A. flavus by cultural and molecular methods (Table 1).

Table 1: Detection of aflatoxigenic and nonaflatoxigenic A. flavus isolates from poultry feed by three methods

Detection method	Total number tested	Positive (%)	Negative (%)
Coconut based medium	25	13	12
detection		52%	48%
Ammonia vapor	25	13	12
detection		52%	48%
PCR results assay	25	17	8
		68%	32%

3.2. Sequencing analysis of PCR product

The sequence analysis of aflR sequences results showed that all the tested sequences were compatible with standard sequences in the NCBI gene bank, (table 2). The first isolate of A.flavus (Af1) showed 100 % homology with the A.flavus ITEM 8083 strain , ID: emb|FN398162.1|strain from NCBI at arange of alignment 284-913. The second isolate (Af2) has 99% homology with A.flavus ITEM 8083 strain , ID: emb|FN398162.1| at arange of alignment 224-940. The third isolate

(Af3) has compatibility 99% homology with the A.flavus strain ITEM 8083 strain, ID: emb|FN398162.1 at arange of alignment 292-1013, the fourth isolate (Af4) showed 100% homology with strain A. flavus ITEM 8083 strain, (ID: emb|FN398162.1 at arange of alignment 592-940 in NCBI and the fifth isolate (Af5) result 100% homology with A. flavus ITEM 8083 strain , ID: emb|FN398162.11 at arange of alignment 597-938.

Table 2:The compatibility of strains of A.flavus with other strains from NCBI.

Isolate	Aligned reference strain	Range of alignment	Percentage of homology
Af1	ITEM 8083 ID: emb|FN398162.1|	284-913	100%
Af2	ITEM 8083 ID: emb|FN398162.1|	224-940	99%
Af3	ITEM 8083 ID: emb|FN398162.1|	292-1013	99%
Af4	ITEM 8083 ID: emb|FN398162.1|	592-940	100%
Af5	ITEM 8083 ID: emb|FN398162.1|	597-938	100%

4. Discussion

DNA extracted from 17 A. flavus isolates . Results showed positive PCR reaction indicating the high specificity of aflR primer. After agarose gel electrophoresis, a band size 798 bp was seen. This result agrees with previous studies of several authors [18, 19, 20, 21]. Only Shapira et al., [22] have described PCR approach for the detection of aflatoxigenic fungi, using the genes ver-1 and omtA as targets whose obtained results with DNA from A. parasiticus, only weak signals were obtained with those of A. flavus with these primer pairs. The results of this study demonstrated that aflR primer based PCR method had high sensitivity and specificity in detecting aflatoxigenic aspergilli in pure culture systems and with strong signals were obtained with aflatoxigenic A. flavus and A. parasiticus. This difference could be due to the different primer pairs used for amplification.

The use of CAM for detection of aflatoxins is not always reliable because of the high sensitivity of Aspergillus to ingredients of the medium [23], so the results of aflatoxigenic detection are not always positive result of determination by CAM is positive by PCR detection based on aflR gene , and vice versa, these result agreed with [24] and [25]. So this detection is not accurate to determine the aflatoxigenic from nonaflatoxigenic A.flavus , but the detection by PCR is more accurate , sensitive , specific , rapid and less laborious than conventional methods to determine aflatoxigenic from nonaflatoxigenic A.flavus [26]. The result by PCR confirmed that 17(68%) from 25 isolates of A.flavus are aflatoxegenic (positive) which can produce aflatoxin and are more accuracy from the result by coconut based medium and ammonia vapor detection.

The compatibility of all analyzed isolates with the same standard type strain of A. flavus ITEM 8083 strain, ID: emb|FN398162.1 could be related to the high similarity of aflR copy with the standard copy of A. flavus ITEM 8083 strain , ID: emb|FN398162.1strain. As this gene is one of the coding genes with very low polymorphism among species. According to the alignment loci difference which may be related to the sequencing process as the noise of sequence which should be discarded from different locations loci according to the part of the sequence that contained the noise.

5. Conclusion

According to above results it can be concluded that more than fifty percent of Aspergillus flavus isolated from poultry feed were aflatoxigenic strains by producing aflatoxins. This feeds can be harmful to birds if used in poultry field.

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