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 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 aflatoxin 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’-AGTGCAAGTTGCTCAGAAACA-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 aflatoxicigenic or non aflatoxicigenic ability of these isolates. These tests revealed that 13 (52%) of isolates were positive aflatoxicigenic and 12(48%) of isolates were negative or nonaflatoxicigenic (Table 1).

3.1. Molecular detection
Twenty five isolates of A. flavus were tested in order to determine either aflatoxicigenic or non aflatoxicigenic isolates. This test confirmed that 17 (68%) were aflatoxicigenic isolates(positive) by PCR and 8(32%) were nonaflatoxicigenic (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           |
| Ammonia vapor detection   | 25                  | 13           | 12           |
| PCR results assay         | 25                  | 17           | 8            |

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 (Afl1) showed 100% homology with the A. flavus ITEM 8083 strain, ID: emb|FN398162.1| strain from NCBI at a range of alignment 284-913. The second isolate (Afl2) has 99% homology with A. flavus ITEM 8083 strain, ID: emb|FN398162.1| at a range 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 range of alignment 292-1013, the fourth isolate (Af4) showed 100% homology with strain A. flavus ITEM 8083 strain, (ID: emb|FN398162.1 at range 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 range 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                | 284-913            | 100%                   |
|         | ID: emb|FN398162.1|                |                        |
| Af2     | ITEM 8083                | 224-940            | 99%                    |
|         | ID: emb|FN398162.1|                |                        |
| Af3     | ITEM 8083                | 292-1013           | 99%                    |
|         | ID: emb|FN398162.1|                |                        |
| Af4     | ITEM 8083                | 592-940            | 100%                   |
|         | ID: emb|FN398162.1|                |                        |
| Af5     | ITEM 8083                | 597-938            | 100%                   |
|         | ID: emb|FN398162.1|                |                        |

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 aflatoxigenic (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|FN398162.1 strain. 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.

References
[1] Krnjaja, V., Stojanović L. j., Cmiljanić R., Trenkovski, S., Tomašević D. 2008. The presence of potentially toxigenic fungi in poultry feed. Biotehnol. Anim. Husb. 24(5-6):87-93. DOI 10.2298/BAH080607KR.
[2] Lanyasunya, T. P., Wamae, L.W., Musa, H. H., Olowofeso, O. and Lokwepeu, I. K. 2005. The risk of mycotoxins contamination of dairy feed and milk on smallholder dairy farms in Kenya. Pak. J. Nutr. 4(3): 162–169.
[3] Whitlow, L. W. and Hagler, Jr W. M. 2008. Mold and mycotoxin issues in dairy cattle:effects, prevention and treatment. WCDS Advances in Dairy Technol, 20:195-209.
[4] Yeni, F., Acar, S., Polat, Ö. G., Soyer, Y. and Alpas, H. 2014. Rapid and standardized methods for detection of foodborne pathogens and mycotoxins on fresh produce. Food Cont. 40: 359–367. DOI 10.1016/j.foodcont.2013.12.020.
[5] Khayoon, W. S., Saad,B., Salleh,B., Manaf, N. H. A. and Latiff, A. 2014. Micro-solid phase extraction with liquid chromatography-tandem mass spectrometry for the determination of aflatoxins in coffee and malt beverage. Food Chemistry 147:287-294.
[6] Milicevic D 2009 Mycotoxin in the food chain – old problems and new solutions. Tehnologija Mesta. 50 (1-2): 99-111.
[7] Al-Kahtani, M. D. F.  2014. Isolation of fungi and their mycotoxin extract from stored wheat and other grains importer in Saudi Arabia. Am. J. Food Technol. 9: 370-376.
[8] Czéh, A. 2014. Multiplexed solid phase immunoassay platform for polymycotoxins: beyond 20th century applications. PhD Thesis, University of Pécs, Hungary.
[9] Dao, H. P., Mathieu, F. and Lebrihi, A. 2005. Two primer pairs to detect OTA producers by PCR method. Intl. J. Food Microbiol. 104 (1):61-7.
[10] Atkins, S. D. and Clark, I. M. 2004. Fungal molecular diagnostics: a mini review. J. Appl. Gen. 45 (1):3-15.
[11] Hussain T., Singh, B. P. and Anwar, F. 2014. A quantitative Real Time PCR based method for the detection of phytophthora infestans causing Late blight of potato, in infested soil. Saudi J. Biol. Sci. 21(4): 380-386. DOI 10.1016/j.sjbs.2013.09.012.
[12] Criseo, G., Bagnara, A. and Bisignano, G. 2001. Differentiation of aflatoxinproducing and non-producing strains of Aspergillus flavus group. Letters in Applied Microbiology, 33(4): 291-295.
[13] Dyer, S. K. and Mccammon, S. 1994. Detection of toxigenic isolates of Aspergillus flavus and related species on coconut cream agar. J. Appl. Bacteriol. 76: 75-78.
[14] Lin, M. T. and Dianese J. C. 1976. A coconut-agar medium for rapid detection of aflatoxin production by Aspergillus spp. Phytopathology 66: 1466-1469.
[15] Davis, N. D., Iyer, S. K. and Diener, U. L. 1987. Improved method of screening for aflatoxin with a coconut agar medium. Appl. Environ. Microbiol. 53(7):1593–1595.
[16] Hara, S., Fennell, D. I. and Hesselteine, C. W. 1974. Aflatoxin-producing strains of Aspergillus flavus detected by fluorescence of agar medium under ultraviolet light. Appl Microbiol. 27(6):1118-1123.
[17] Saito, M. and Machida, S. 1999. A rapid identification method for aflatoxin producing strains of Aspergillus flavus and A. parasiticus by ammonia vapor. Mycoscience 40 (2):205-208. DOI 10.1007/BF02464300.
[18] Manonomani H K, Anand S., Chandrashekar, A and Rati, E R 2005. Detection of aflatoxigenic fungi in selected food commodities by PCR. Process Biochemistry 40 (8):2859–2864. DOI 10.1016/j.procbio.2005.01.004.
[19] http://blast.ncbi.nlm.nih.gov/Blast.cgi.
[20] Fauber, P., Geisen, R. and Holzapfe, W. H. 1997. Detection of aflatoxigenic fungi in figs by a PCR reaction. Intl. J. Food Microbiol. , 36(2-3):215–220.
[21] Noorbakhshh, R., Bahrami, A. R., Mortazavi, S. A., Forghani, B. and Bahreini, M. 2009. PCR-based identification of aflatoxigenic fungi associated with Iranian saffron. Food Sci.
[22] Hasham, A., Al-Kazaz, A., Abdulmalek, H. W. 2013. PCR detection of Aspergillus flavus isolates for aflatoxin B1 producer. *J. Biotechnol. Res. Center* 7(3):81-89.

[23] Shapira, R., Pasti, N.R., Fyai, O., Minastherov, M., Mett, A. and Salomon, R. 1996. Detection of aflatoxigenic molds in grains by PCR. *Appl. Environ Microbiol.* 62(9):3270–3273. DOI 10.1128/AEM.62.9.3270-3273.1996.

[24] Yazdani, D., Zainal Abidin, M. A., Tan, Y. H. and Kamaruzaman, S. 2010. Evaluation of the detection techniques of toxigenic Aspergillus isolates. *Afr. J. Biotechnol.* 9 (45): 7654-7659.

[25] Abdel-Hadi, A., Carter, D. and Magan, N. 2011. Discrimination between aflatoxigenic and non-aflatoxigenic strains of Aspergillus section Flavi group contaminating Egyptian peanuts using molecular and analytical techniques. *World Myco. J.* 4(1): 69-77.

[26] Shweta, S., Madhavan, S., Paranidharan, V. and Velazhahan, R. 2013. Detection of Aspergillus flavus in maize kernels by conventional and realtime PCR assays. *Int. Food Res. J.* 20(6): 3329-3335.