Chapter 19

Highly Sensitive PCR-Based Detection Specific to *Aspergillus flavus*

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Abstract

Aspergillus flavus is an important fungal species that frequently contaminates food commodities with diverse toxins, with aflatoxins being the most relevant in food safety. In addition, this is one of the major pathogenic *Aspergillus* species. In this work, specific PCR-based protocol for this species is described which allows the discrimination of other closely related species from the *Aspergillus* section *Flavi*, particularly *Aspergillus parasiticus*. The specific primers were designed on the multicopy internal transcribed region of the rDNA unit (ITS1-5.8S-ITS2 rDNA).

Key words: *Aspergillus flavus*, Aflatoxin, Polymerase chain reaction, Detection, Internal transcribed spacer

1. Introduction

Aflatoxins are potent carcinogenic, mutagenic, and teratogenic secondary metabolites and are produced predominantly by *Aspergillus flavus* and *Aspergillus parasiticus* (1, 2). Currently, upper limits of aflatoxins in foodstuffs are under regulation in the European Union (3). This regulation often results in the destruction of contaminated agricultural products, causing significant economic losses. Aflatoxigenic fungi can contaminate several food commodities including cereals (4), peanuts (5), spices (6), and figs (7, 8). In addition, *A. flavus*, together with *Aspergillus fumigatus*, are responsible for 90% of aspergillosis in human beings (9).

The level of mold infestation and the identification of governing species are important indicators of the quality of the raw material, and predict the potential risk for the presence of mycotoxins (10).

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Early detection of aflatoxin-producing species is crucial to prevent aflatoxins entering the food chain. PCR-based methods that target DNA are considered a good choice for rapid diagnosis because of their high specificity and sensitivity, especially when multicopy target sequences are used (11). This PCR protocol is based on multicopy sequences (ITS of rDNA units) specific to *A. flavus*, and allows a rapid, very efficient, and sensitive detection of this fungus.

2. Materials

2.1. DNA Extraction

1. DNeasy Plant Mini Kit (Qiagen). Store at room temperature (15–25°C), except RNase A and AW buffer (at 4°C), and AP1 (at 28°C to avoid precipitation of components).
2. Ethanol (96–100%). Store at room temperature.

2.2. PCRs

1. Primers FLA1 (5′-GTAGGGTTCCTAGCGAGCC-3′) and FLA2 (5′-GGAAAAAGATTGATTTGCGTTC-3′) (Sigma-Aldrich): prepare aliquots of each primer at 20 μM in sterile water and store at –20°C.
2. PCR buffer (10×), $MgCl_2$ solution (50 mM), and Taq DNA polymerase (5 U/mL) (Biotools). Store at –20°C.
3. dNTPs solution (100 mM) (Biotools). Store at –20°C.
4. Molecular biology grade water (MoBio, Carlsbad, USA).
5. Template DNA (10–200 ng). Store at –20°C.

2.3. Agarose Gel Electrophoresis

1. TAE buffer (1×): Tris–acetate 40 mM and EDTA 1.0 mM. Store at room temperature.
2. Agarose D-1 Low EEO (Pronadisa, Madrid, Spain). Store at room temperature.
3. 1% Ethidium bromide solution (Applichem). Store at room temperature.
4. Loading buffer: 25 mg bromophenol blue, 4 g sucrose, and H_2O to 10 mL. Store at 4°C.
5. 100-bp DNA molecular mass marker (MBI Fermentas, Vilnius, Lithuania). Store at 4°C.

3. Methods

3.1. DNA Extraction

1. These instructions assume the extraction of DNA from pure fungal culture (see Note 1), contaminated plant material, or food matrix (see Note 2). The DNeasy Plant Mini Kit includes RNase A enzyme, buffers, columns, and collection tubes.

2. Prepare in advance all the materials required for the protocol: sterilize all material (tips, mortar and pestle, microcentrifuge tubes, etc.); add the appropriate amount of ethanol to buffer AW and AP3/E, as indicated on the bottle, to prepare a working solution; preheat a water bath to 65°C; and label each microcentrifuge tube and column.
3. Grind the plant, food matrix, or fungal tissue under liquid nitrogen using a sterile mortar and pestle (see Note 3). Transfer the tissue powder (a maximum of 100 mg) to a 1.5-mL microcentrifuge tube.
4. Add 400 µL of buffer AP1 (lysis buffer) and 4 µL of RNase A stock solution (100 mg/mL) and vortex vigorously.
5. Incubate the mixture for 10 min at 65°C to lyse the cells. Mix two or three times during incubation by inverting the tube.
6. Add 130 µL of buffer AP2 (precipitation buffer, see Note 4) to the lysate, mix, and incubate for 5 min in ice to precipitate detergent, proteins, and polysaccharides present in the sample.
7. Centrifuge for 6.5 min at 16,000 × *g*.
8. Transfer the supernatant into the QIAshredder Mini spin column (lilac) placed in a 2-mL collection tube, and centrifuge for 2.5 min at 16,000 × *g*.
9. Transfer the flow-through fraction (usually about 430 µL) from step 8 into a new tube without disturbing the cell debris pellet.
10. Add 1.5 volume (usually about 645 µL) of buffer AP3/E (binding buffer) to the cleared lysate, and mix immediately by pipetting.
11. Pipette 650 µL of the mixture from step 10, including any precipitate that may have formed, into the DNeasy Mini spin column placed in the 2-mL collection tube. Centrifuge for 1 min at 6,000 × *g* and discard the flow-through. Reuse the collection tube in step 12.
12. Repeat step 11 with remaining sample. Discard the flow-through and collection tube.
13. Place the DNeasy Mini spin column into a new 2-mL collection tube, add 500 µL of buffer AW (wash buffer), and centrifuge for 1 min at 6,000 × *g*. Discard the flow-through and reuse the collection tube in step 14.
14. Add 500 µL of buffer AW to the DNeasy Mini spin column and centrifuge for 2.5 min at 16,000 × *g* to dry the membrane.
15. Transfer the DNeasy Mini spin column to a 1.5-mL or 2-mL microcentrifuge tube and add 100 µL of molecular degree water directly onto the DNeasy membrane. Incubate for

5 min at room temperature and then centrifuge for 1 min at 6,000 × *g* to elute.

16. Measure the amount of template DNA spectrophotometrically. The concentration and purity of DNA can be determined by measuring the absorbance at 260 nm (A260) and 280 nm (A280). Purity is determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA has an A260/A280 ratio of 1.8–2.0.
17. Store the DNA at –20°C.

3.2. PCR Amplification

1. Mix the following components in a 0.5-mL microcentrifuge tube: 2 µL (10–200 ng total DNA amount, see Note 5) of template DNA (see Note 6), 1 µL of each primer FLA1 and FLA2, 2.5 µL of 10× PCR buffer, 1 µL of $MgCl_2$, 0.2 µL of dNTPs, and 0.15 µL of Taq DNA polymerase (see Note 7). Add molecular biology grade water up to 25 µL.
2. It is recommended to perform control PCR assays (see Note 8).
3. The PCR amplification protocol is 1 cycle of 5 min at 95°C; 26 cycles of 30 s at 95°C (denaturalization), 30 s at 58°C (annealing), and 45 s at 72°C (extension); and finally, 1 cycle of 5 min at 72°C.
4. Store the PCR products at 4°C.

3.3. Agarose Gel Electrophoresis

1. These instructions are for making a 10 × 15 cm 1% agarose gel using a Bio-Rad Wide Mini-Sub Cell GT gel system.
2. Weigh out 0.6 g of agarose into a 250-mL conical flask. Add 60 mL of 1× TAE and swirl to mix.
3. Microwave the mixture for about 1 min to dissolve the agarose.
4. Leave it to cool down to about 60°C (just hot to hold in bare hands).
5. Add 1 µL of ethidium bromide (10 mg/mL) and swirl to mix (see Note 9).
6. Pour the gel slowly into the tray. Insert the comb and check that it is correctly positioned. The gel should polymerize within 20 min.
7. Pour 1× TAE buffer into the gel tank to submerge the gel to 2–5 mm depth. This is the running buffer.
8. Prepare the samples by adding 5 µL of loading buffer to a 15 of each sample into an appropriate labeled tube.
9. Once the gel has set, remove the comb carefully, and load 20 µL of each sample in a well. Reserve one well for the DNA molecular mass marker.

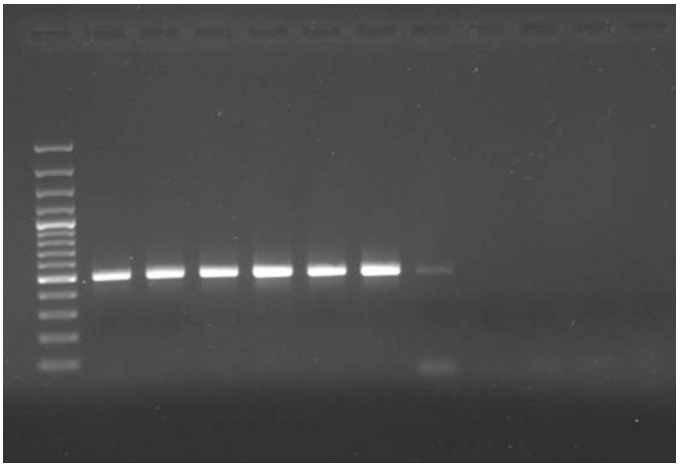

Fig. 1. PCR amplification using primers FLA1/FLA2 and DNA from Lanes 1 and 2: *Aspergillus flavus* strains (CECT 2681, ITEM 4591); Lanes 3 and 4: DNA from wheat flour contaminated with *A. flavus*; Lane 5: DNA from barley flour contaminated with *A. flavus*; Lanes 6 and 7: DNA from paprika contaminated with *A. flavus*; Lane 8: Non-template control; Lane 9: *Aspergillus parasiticus* Cab5dch6; Lane 10: *Aspergillus bombycis* IHEM 19289; Lane 11: *Aspergillus carbonarius* CECT 2086. M: 100-bp DNA molecular mass marker.

10. Complete the assembly of the gel unit and connect to a power supply. Run the gel at 80 V for about 45 min while monitoring the progress of the gel with reference to the marker dye.
11. The gel is then placed in an UV-light box to visualize the PCR products. An example of the results produced is shown in Fig. 1.

4. Notes

1. Fungal DNA can be obtained from isolates cultured in 100-mL Erlenmeyer flasks containing 20 mL of liquid Sabouraud medium (Scharlau Chemie, Barcelona, Spain) and incubated at 28°C and 150 rpm. *A. flavus* mycelia from 3-day-old cultures can be harvested by filtration through Whatman No. 1 paper and frozen in liquid nitrogen. Mycelia can be stored at –80°C until DNA extraction.
2. This protocol can be adapted for many other systems, using instead other more appropriate DNA extraction kits, e.g., DNeasy Blood & Tissue kit (Qiagen).
3. The primary disruption of the tissue is an essential step that must be performed carefully. It can be performed with another disruption method, such as commercially available homogenizers.

4. Phenolic compounds present in some food matrices, such as grapes or pepper, might interfere with DNA extraction procedure or inhibit PCR assay. In these cases, polyvinylpyrrolidone (0.33%) (Sigma-Aldrich) can be added to AP2 buffer in order to remove these compounds.
5. When the assay is performed from a fungal pure culture, the total amount of DNA obtained is normally between 10 and 50 ng. When PCR assay is performed with DNA from a food matrix or a plant tissue, the total amount of DNA per reaction should be between 150 and 200 ng.
6. Positive (with 2 μL of *A. flavus* pure DNA) and negative (with 2 μL of molecular biology grade water instead of DNA template) controls must be included in PCR assays.
7. Use filter tips to prepare PCR. Taq polymerase must be maintained in ice during its manipulation.
8. It is recommended to test the DNA integrity by performing a control PCR with the universal fungal primers ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATG 3′), using the amplification program described by Henry et al. (12).
9. Ethidium bromide is a potent mutagenic agent and must be handled with care; always wear a suitable laboratory coat and disposable gloves. All ethidium bromide waste must be disposed in appropriate containers.

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