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Development of a PCR Protocol To Detect Aflatoxigenic Molds in Food Products

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ABSTRACT

Aflatoxins are secondary metabolites produced mainly by *Aspergillus* species growing in foodstuffs. Because aflatoxins have important health effects, the detection of early contamination of foods by aflatoxigenic molds should be useful. In the present work, a reliable conventional PCR method for detecting aflatoxigenic molds of various species was developed. Fifty-six aflatoxigenic and nonaflatoxigenic strains commonly reported in foodstuffs were tested. Aflatoxin production was first confirmed by micellar electrokinetic capillary electrophoresis or/and high-pressure liquid chromatography–mass spectrometry. Based on the conserved regions of the *O*-methyltransferase gene (*omt-1*) involved in the aflatoxin biosynthetic pathway, six primer pairs were designed. With only the designed primer pair AFF1-AFR3, the expected PCR product (381 bp) was obtained in all of the tested aflatoxigenic strains of various species and genera. Amplification products were not obtained with this primer pair for any of the nonaflatoxigenic reference molds. However, an amplicon of 453 bp was obtained for all aflatoxigenic and nonaflatoxigenic mold reference strains with a PCR protocol based on the constitutive fungal β-tubulin gene, which was used as a positive fungal control. The PCR protocol based on *omt-1* detected as little as 15 pg of DNA from aflatoxigenic molds and 10^2 to 10^3 CFU/g in contaminated food samples. This PCR protocol should be used as a routine technique to detect aflatoxigenic molds in foods.

Aflatoxins are a group of polyketide-derived furanocoumarins possessing hepatocarcinogenic, immunosuppressive, carcinogenic, teratogenic, and mutagenic properties *(20, 26, 31, 35)*. These fungal toxins are common contaminants of a wide variety of foods such as nuts *(11, 48)*, figs *(33)*, spices *(4, 12)*, maize *(14)*, wheat *(16)*, and melon *(5, 6)*. The presence of aflatoxin residues in food of animal origin such as meat, milk, eggs, and cheese may be the result of contamination *(22, 46)*. Aflatoxins have been detected in processed foods of animal origin, such as meat products, because of fungal growth *(21, 38)*.

In food products, aflatoxins are produced mainly by *Aspergillus flavus (7, 8)* and *Aspergillus parasiticus (2, 37)*. Other species of *Aspergillus,* including *A. toxicarius, A. tamarii*, and *A. versicolor,* also can produce aflatoxins *(1, 3, 40)*.

To prevent aflatoxins from entering the food chain, the detection of aflatoxigenic molds in the food products is a useful strategy. Traditional methods, which are based mainly on morphological features of toxigenic molds, can be used for detection, but they are time-consuming and require considerable expertise. In contrast, PCR techniques are suitable for rapid and sensitive detection of molds that can produce aflatoxins in foods *(28, 50)*. Most of the primers designed for these PCR methods rely on genes directly involved in the biosynthesis of aflatoxins such as the aflatoxin regulatory gene (*aflR*) *(28, 44, 50)*, the versicolorin A dehydrogenase gene (*ver-1*) *(15, 43, 50)*, the sterigmatocystin *O*-methyltransferase gene (*omt-1*) *(43, 44)*, the norsolorinic acid reductase gene (*nor-1*) *(15)*, the aflatoxin biosynthesis gene cluster of *A. flavus* (C2) *(9)*, and a regulatory gene cloning of the *A. parasiticus* gene (APA) *(43)*. Although current PCR methods target some specific aflatoxigenic *Aspergillus* species, especially *A. flavus* and *A. parasiticus,* no protocols have been developed to detect a wider range of aflatoxigenic mold species, including those belonging to other genera.

To develop a reliable PCR protocol to detect aflatoxigenic molds in foods, factors to be considered include the complexities of food matrices *(19)* and the fact that some of their components can remain in final DNA extracts, causing inhibition of the PCR amplification. Consequently, a PCR protocol designed to detect aflatoxigenic molds in food samples should be evaluated for its sensitivity in various food matrices.

The aim of this study was to develop a PCR method for detecting aflatoxigenic mold strains of various fungal species and genera that usually contaminate food products. The sensitivity of the method in foods also was analyzed.

MATERIALS AND METHODS

Mold strains and culture conditions. The 56 reference mold strains used in this study are listed in Table 1. The isolates were

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TABLE 1. *Aflatoxin production by reference mold strains and PCR results with primer pairs AFF1-AFR3 and Bt2a-Bt2b for the sterigmatocystin O-methyltransferase and β-tubulin genes, respectively*[a]

Species	Strain[b]	Aflatoxin production[c]		PCR results	
		Reported by culture collection	Detected by MECE and/or HPLC-MS	AFF1-AFR3[d]	Bt2a-Bt2b[e]
Aspergillus flavus	**CECT 2687**	**+**	**+**	**+**	**+**
A. flavus	**IBT 3696**	**+**	**+**	**+**	**+**
A. flavus	**CECT 2684**	**U**	**+**	**+**	**+**
A. flavus	**CBS 573.65**	**+**	**+**	**+**	**+**
A. flavus	**CBS 120.62**	**+**	**+**	**+**	**+**
A. parasiticus	**CECT 2688**	**U**	**+**	**+**	**+**
A. parasiticus	**CECT 2682**	**+**	**+**	**+**	**+**
A. parasiticus	**CECT 2681**	**+**	**+**	**+**	**+**
A. parasiticus	**CBS 571.65**	**+**	**+**	**+**	**+**
A. versicolor	CECT 2664	U	−	−	+
A. versicolor	CECT 2903	U	−	−	+
A. versicolor	**CECT 2814**	**U**	**+**	**+**	**+**
A. oryzae[f]	**CECT 2094**	**U**	**+**	**+**	**+**
A. oryzae[g]	**CECT 2095**	**U**	**+**	**+**	**+**
A. tamarii	**CBS 575.65**	**U**	**+**	**+**	**+**
A. tamarii	**CBS 109.63**	**U**	**+**	**+**	**+**
A. tubingensis	CECT 20543	U	−	−	+
A. tubingensis	**CECT 20545**	**U**	**+**	**+**	**+**
A. niger	CECT 20157	U	−	−	+
A. ochraceoroseus	**CBS 101.887**	**U**	**+**	**+**	**+**
A. awamori	CBS 101702	U	−	−	+
A. foetidus	**CBS 101.708**	**U**	**+**	**+**	**+**
A. ochraceus	**CBS 589.68**	**U**	**+**	**+**	**+**
A. terreus	CBS 601.65	U	−	−	+
Rhizopus oryzae	**CBS 607.68**	**+**	**+**	**+**	**+**
Emericella heterothallica	CBS 488.65	U	−	−	+
E. nidulans	CBS 465.65	U	−	−	+
E. quadrilineata	CBS 235.65	U	−	−	+
Penicillium viridicatum	CECT 2320	U	−	−	+
P. aurantiogriseum	CECT 2918	U	−	−	+
P. aurantiogriseum	**CECT 2264**	**U**	**+**	**+**	**+**
P. commune	CBS 247.32	U	−	−	+
P. polonicum	FHSC-2	U	−	−	+
P. polonicum	CBS 112490	U	−	−	+
P. polonicum	CBS 639.95	U	−	−	+
P. polonicum	CBS 101479	U	−	−	+
P. dipodomyicola	IBT 26223	U	−	−	+
P. dipodomyicola	CBS 110425	U	−	−	+
P. dipodomyicola	CBS 110426	U	−	−	+
P. expansum	CECT 2278	U	−	−	+
P. expansum	CECT 20140	U	−	−	+
P. expansum	CECT 2279	U	−	−	+
P. expansum	CECT 2280	U	−	−	+
P. griseofulvum[h]	**IBT 14319**	**U**	**+**	**+**	**+**
P. griseofulvum	CBS 485.84	U	−	−	+
P. griseofulvum	CBS 110420	U	−	−	+
P. griseofulvum	CECT 2919	U	−	−	+
P. camemberti	CECT 2267	U	−	−	+
P. camemberti	CBS 273.97	U	−	−	+
P. verrucosum	FHSC-3	U	−	−	+
P. verrucosum	CECT 2906	U	−	−	+
P. verrucosum	CBS 323.92	U	−	−	+
P. melanoconidium	CBS 64195	U	−	−	+
P. melanoconidium	CBS 109605	U	−	−	+
P. nordicum	CBS 110769	U	−	−	+
P. carneum	CBS 468.95	U	−	−	+

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grown at 25°C on potato dextrose agar (PDA; Scharlau Chemie S.A., Barcelona, Spain) for 20 days and then stored as a conidial suspension in 10% (vol/vol) glycerol (Scharlau) at −80°C until used.

Determination of aflatoxin production. Conidia of each of the 56 mold strains listed in Table 1 were inoculated at three points on PDA plates and incubated for 15 days at 25°C. The contents of three petri dishes were extracted with chloroform, filtered twice through anhydrous sodium sulfate, and evaporated in a rotatory evaporator at 40°C as previously described by Núñez et al. *(32)*. The residue was resuspended in 5 ml of chloroform, filtered through a 0.45-mm-pore-size nylon membrane (Micron Separation Inc., Westborough, MA), and evaporated to dryness under a gentle stream of nitrogen. The extracts were stored at 4°C in the dark until used and resuspended in 200 μl of acetonitrile just before analysis. Aflatoxin production was analyzed by micellar electrokinetic capillary electrophoresis (MECE) and high-pressure liquid chromatography–mass spectrometry (HPLC-MS).

MECE was carried out according to Martín et al. *(29)* in a P/ACE 5500 unit with a photodiode array detector (Beckman Instruments, Fullerton, CA). A fused silica capillary 57 cm long and 75 mm inside diameter was used for separation with 25 mM sodium tetraborate and 50 mM sodium dodecyl sulfate (pH 9) as the running buffer at 15 kV, a maximum current of 200 mA, and a capillary cassette temperature of 23°C. The absorbance was recorded at 200 and 280 nm wavelengths. For each peak, a spectrum of absorbance between 190 and 600 nm was obtained with the photodiode array detector.

HPLC-MS was performed according to Núñez et al. *(32)* in an HP series 1100 apparatus (Hewlett Packard, Palo Alto, CA). A Supelcosil LC-18 column (SUPELCO, Bellefonte, PA) was used with mobile phases of 100% water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) in a gradient from 10 to 99% B. Aflatoxins were identified in an LCQ Mass Spectrometer (Finnigan, San Jose, CA) with an atmospheric pressure chemical ionization source, according to the retention time and molecular mass as stipulated by the manufacturer.

In both MECE and HPLC-MS analyses, aflatoxins B1, B2, G1, and G2 (Sigma Aldrich Co., St. Louis, MO) were used as standards.

DNA extraction from pure cultures. Mold strains were inoculated using the three-point method onto PDA plates and incubated for 4 days at 25°C. About 50 mg of mycelium was scraped off the PDA plate and used for DNA extraction following the method developed by Sánchez et al. *(41)*. The mycelium was digested with proteinase K (1 mg/ml; Sigma Aldrich) and lyticase (4 U/ml; Sigma Aldrich), frozen in liquid nitrogen, and ground in a sterilized precooled mortar and pestle. DNA was extracted in a semiautomatic vacuum system (Prism 6100 Nucleic Acid PrepStation, Applied Biosystems, Foster City, CA).

The DNA concentration was determined in 1% (wt/vol) agarose gels by submerged electrophoresis with 1× Tris-acetate-EDTA (TAE) buffer at 90 V for 15 min. Gels were stained with ethidium bromide (0.5 μg/ml; Sigma Aldrich) for 20 min, and the DNA bands were visualized by UV transillumination (G-Box, Syngene, Synoptics Group, Frederick, MD). Gels were photographed with a GeneSnap camera (Syngene). A standard equation relating the DNA concentration standards (0.25 μg/μl; Roche Diagnostics, Indianapolis, IN) and their absorbance values obtained with the GeneTools analysis software (Syngene) was then generated to estimate the concentration (in nanograms) of purified DNA. The quality of the extracted DNA was determined spectrophotometrically in a Biophotometer (Eppendorf AG, Hamburg, Germany) based on the ratio of 260:280.

PCR primers and PCR amplification conditions. Genomic DNA from pure cultures of *A. flavus* CECT 2687, *A. parasiticus* CECT 2688, *A. parasiticus* CECT 2682, *A. flavus* IBT 3696, and *Aspergillus oryzae* CECT 2094 was used for PCR with previously reported primer pairs *(9, 15, 28, 36, 43)*. A negative control with DNA from the nonaflatoxigenic *Penicillium viridicatum* CECT 2320 as the template was included. All assays were performed in triplicate.

The primer pair that produced specific amplification products from all tested aflatoxigenic mold strains was selected for further analysis. The PCR products obtained with the primer pair OMT-F–OMT-R *(36)* were purified with the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and submitted for sequencing at the Instituto de Biomedicina (Consejo Superior de Investigaciones Científicas, Valencia, Spain). To test the specificity, the obtained sequences were compared with the *omt-1* sequences stored in the NCBI database (GenBank accession no. L25835.1; www.ncbi.blast.com). These sequences were then analyzed and aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to design six new primers from conserved regions using the Primer Express software (Applied Biosystems) (Table 2). These primers were tested with DNA from 12 aflatoxigenic reference molds (*A. flavus* CECT 2687, *A. parasiticus* CECT 2688, *A. parasiticus* CECT 2682, *A. flavus* IBT 3696, *A. parasiticus* CECT 2681, *Aspergillus tubingensis* CECT 20545, *A. flavus* CBS 573.65, *A. flavus* CBS 120.62, *Aspergillus ochraceoroseus* CBS 101.887, *A. parasiticus* CBS 571.65, *A. versicolor* CECT 2814, and *Aspergillus foetidus* CBS 101.708) to select the most specific primer pair. Amplification reactions were performed in a total volume of 50 μl containing 5 μl of 10× Mg-free PCR buffer, 1.5 μl of 50 mM $MgCl_2$, 1 μl of 10 mM concentration of the deoxynucleoside triphosphate mix, 5 μl of 10 mM concentrations of each primer, 1.25 μl of 2 U/μl *Taq*

←

[a] Mold strains that produced aflatoxin are shown in bold.
[b] CECT, Spanish Type Culture Collection; IBT, Type Culture Collection of the Department of Biotechnology, Technical University of Denmark; CBS, Centraalbureau voor Schimmelcultures, The Netherlands; FHSC, Food Hygiene and Safety, University of Extremadura, Spain.
[c] +, positive for aflatoxin production; U, aflatoxin production unknown; −, negative for aflatoxin production. MECE, mycellar electrokinetic capillary electrophoresis; HPLC-MS, high-pressure liquid chromatography–mass spectrometry.
[d] Detection of 381-bp amplicon with primer pair AFF1-AFR3.
[e] Detection of 453-bp amplicon with primer pair Bt2a-Bt2b.
[f] Identified as *A. flavus* based on analysis of a partial sequence of the ITS region and the β-tubulin gene.
[g] Identified as *A. parasiticus* based on analysis of a partial sequence of the ITS region and the β-tubulin gene.
[h] Identified as *P. commune* based on analysis of a partial sequence of the ITS region and the β-tubulin gene.

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TABLE 2. *PCR primer pairs designed in this study derived from the sterigmatocystin* O-*methyltransferase gene* (omt-1) *and PCR conditions*

Primer name	Primer sequence (5′ to 3′)	PCR conditions: Initial denaturation	PCR conditions: No. of cycles	Cycle conditions: Denaturation	Cycle conditions: Annealing	Cycle conditions: Extension	Final extension	Position[a]	Amplicon size (bp)
AFF1	CTTCGAGGATGTGCCCAGCGC	94°C, 10 min	33	95°C, 1 min	60°C, 2 min	72°C, 2 min	72°C, 5 min	1,103	458
AFR1	CCAGTGGCTTCGTGGCTTCG							1,542	
AFF1	CTTCGAGGATGTGCCCAGCGC	94°C, 10 min	33	95°C, 1 min	60°C, 1 min	72°C, 2 min	72°C, 5 min	1,103	429
AFR2	GGCTTCGTGGCTTCGGTGCC							1,513	
AFF1	CTTCGAGGATGTGCCCAGCGC	94°C, 5 min	30	94°C, 1 min	58°C, 2 min	72°C, 1.5 min	72°C, 5 min	1,103	381
AFR3	CGAACCTCGTCCACAGTGC							1,466	
AFF1	CTTCGAGGATGTGCCCAGCGC	94°C, 10 min	30	94°C, 1 min	58°C, 2 min	72°C, 1.5 min	72°C, 5 min	1,103	505
AFR4	ACCACGACCGCCGCC							1,594	
AFF2	ATTCATGCCTTGGTTGGATT	94°C, 5 min	30	94°C, 1 min	58°C, 2 min	72°C, 1.5 min	72°C, 5 min	1,195	289
AFR3	ATTCATGCCTTGGTTGGATT							1,466	
AFF1	CTTCGAGGATGTGCCCAGCGC	94°C, 10 min	30	94°C, 1 min	58°C, 2 min	72°C, 2 min	72°C, 5 min	1,103	449
AFR5	CAAACATCTCCTCTACCAGTGGC							1,530	

[a] Positions in accordance with the published sequences of the *omt-1* gene of *Aspergillus flavus* (GenBank accession no. L25835).

polymerase (Finnzymes, Espoo, Finland), and 5 μl (10 ng) of genomic DNA. The amplification reactions were carried out in a programmable thermal cycler (Mastercycler epgradient, Eppendorf AG) under the PCR conditions described in Table 2. A negative control with DNA from the nonaflatoxigenic *P. viridicatum* CECT 2320 as the template was included in each PCR assay.

A 5-μl aliquot of each amplification product was examined in 2% (wt/vol) agarose gels using 1× TAE buffer at 85 V for 1 h. Gels were stained with ethidium bromide for 20 min, and the products were visualized under UV light and photographed as described above. A DNA molecular size marker of 2.1 to 0.15 kbp (Roche Diagnostics) was used to determine the size of the PCR products. The specificity of the selected PCR protocol for the detection of aflatoxigenic molds was evaluated in triplicate with the 56 reference mold strains.

The presence of fungal DNA was tested by PCR designed on the universal fungal β-tubulin gene with primers Bt2a and Bt2b *(17)*. The amplification program was 5 min at 94°C, 32 cycles of 1 min at 94°C, 1 min at 68°C, and 1 min at 72°C, and finally 5 min at 72°C.

Sensitivity of PCR protocol. The sensitivity of the method was assayed by using several concentrations (50 ng to 0.05 pg) of DNA from the aflatoxigenic *A. parasiticus* CBS 571.65 and by amplifying DNA from this strain in the presence of DNA from a nonaflatoxigenic strain. Thus, 50 ng of DNA from *P. viridicatum* CECT 2320 was mixed with various amounts of DNA (5 ng to 5 pg) from the aflatoxigenic strain. The detection limit of the PCR method was defined as the lowest concentration of *A. parasiticus* CBS 571.65 DNA that produced an amplification product.

The sensitivity of the PCR method also was assayed with DNA extracted from three nonsterile groups of foods (cooked products, ripened foods, and nuts and fruit) inoculated with various concentrations of conidia of aflatoxigenic strains. Cooked turkey breast, cooked ham, and mortadella were inoculated with *A. parasiticus* CECT 2682; dry-cured ham, dry-fermented sausage (salchichón), and ripened cheese were inoculated with *Penicillium griseofulvum* IBT 14319; and dried fig, almond, and pistachio were inoculated with *A. flavus* IBT 3696. The selection of mold strains for the inoculation of each food group was based on the most common species found in each group. All assays were carried out in triplicate.

The conidia of each mold strain used for inoculation were extracted by flooding three 20-day-old PDA plates with 5 ml of sterile deionized water containing 10% (vol/vol) glycerol and rubbing the surface with a glass rod. The concentration of conidia in the suspensions was measured by microscopy with a Neubauer counting chamber. Two hundred microliters of decimal dilutions of conidia was used to inoculate 5 g of each food product to produce final inoculation levels of 10^6 to 10 conidia per g. Ten milliliters of Tris-HCl buffer (pH 8.0) was then added to the inoculated food samples, and the resulting mixture was homogenized in a filter bag (BagPage, Interscience, Paris, France) in a stomacher (IUL Instruments, Barcelona, Spain). To directly estimate the load of the inoculated molds, 10-fold dilutions from the obtained filtrate solutions were prepared in 0.1% sterile peptone in pure water. A 0.1-ml volume of these solutions was plated on PDA and incubated at 25°C for 4 days to calculate the CFU per gram in the corresponding foods. The natural fungal contamination of the samples was lower than 10 CFU/g, and characterization of the isolates from noninoculated foods revealed no colonies typical of *A. parasiticus, P. griseofulvum,* or *A. flavus*.

For DNA extraction, the remaining volume of each filtrate solution was transferred to a sterile tube and centrifuged at

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2,500 rpm for 30 s and then at 13,000 rpm for 10 min in a 5417R centrifuge (Eppendorf AG, Hamburg, Germany). The supernatant was removed, and the pellet was resuspended in 100 μl of previously heated (95°C for 10 min) sterile deionized water. After cooling this solution on ice for 10 min, 200 μl of cetyltrimethylammonium bromide (CTAB) extraction buffer (20 g/liter CTAB, 1.4 M NaCl, 20 mM Na_2EDTA, 0.1 M Tris-HCl, pH 8.0), 400 μl of PBS buffer (0.15 M NaCl, 0.01 M sodium phosphate buffer, 0.05% Tween 20, pH 7.4), 20 μl of 10 mg/ml proteinase K, and 200 μl of 4 U/ml lyticase were added, and the mixture was incubated at 65°C for 1 h. The sample was then washed with 600 μl of chloroform and centrifuged at 13,000 rpm for 20 min (5417R centrifuge, Eppendorf AG). The supernatant was transferred to a new tube, 20 μl of RNase A solution (10 mg/ml; Sigma Aldrich) was added, and the tubes were incubated at 37°C for 1 h. A 600-μl volume of chloroform was then added, and the tubes were vortexed and centrifuged at 13,000 rpm for 5 min (5417R centrifuge, Eppendorf AG). The aqueous phase was processed according to the EZNA Fungal DNA Mini Kit protocol (Omega Bio-tek, Doraville, GA), starting with DNA precipitation by adding 600 μl of cold isopropanol. DNA was eluted in 25 μl of previously warmed (65°C) elution buffer and kept at −20°C until used as a template for PCRs.

Three replicates of 5 μl of DNA extracted from each inoculated food were assayed per PCR. Noninoculated negative controls were included in each assay. Results were then visualized on 2% agarose gels as described previously. The detection limit of the PCR method in these food samples was estimated as the lowest count (CFU per gram) of the aflatoxigenic molds in each tested food that produced the specific amplicon of 381 bp. The presence of fungal DNA was tested in all samples by PCR based on the β-tubulin gene following the procedure described above.

The influence of food components on the extraction of mold DNA and the subsequent PCRs was evaluated by using 5 μl of pure DNA from aflatoxigenic *A. parasiticus* CECT 2682, *P. griseofulvum* IBT 14319, and *A. flavus* IBT 3696 mixed in various volumes (1, 2, 3, 4, and 5 μl) of DNA obtained from each corresponding noninoculated food. The PCR products resulting from the amplification reactions were compared with those obtained by amplifying pure DNA from aflatoxigenic mold strains.

Statistical analysis. All the statistical analyses were performed with SPSS v. 15.0. A one-way analysis of variance was carried out to determinate significant differences within and between groups. Tukey's test was used to compare the mean values. Statistical significance was set at $P \leq 0.05$.

RESULTS

Evaluation of aflatoxin production. MECE and HPLC-MS analyses confirmed aflatoxin production in the eight mold strains that had been designated by their respective culture collections as aflatoxigenic (Table 1). In addition, 13 strains with no information on aflatoxin production provided by the culture collections produced this type of mycotoxin (Table 1), in all cases, the aflatoxins were B1 and G1. The remaining 35 strains did not produce detectable amounts of aflatoxins, as determined by MECE or HPLC-MS (Table 1).

PCR detection of aflatoxigenic molds. Several previously reported primer pairs designed from different genes involved in the aflatoxin biosynthesis pathway were tested in the present study. PCR amplification products from DNA of all of the tested aflatoxigenic mold strains were obtained only with primer pair OMT-F–OMT-R (data not shown). The 1,254-bp amplicons generated with these primers were sequenced and analyzed to design six new primers (Table 2). All had 100% similarity with the *omt-1* gene sequences published in the NCBI database (GenBank accession no. L25835).

Primer pair AFF2-AFR3 produced the expected amplification product from 11 of the 12 tested aflatoxigenic strains (Fig. 1a), whereas primer pairs AFF1-AFR1, AFF1-AFR2, and AFF1-AFR5 produced single amplicons of the expected size for only 8 of these strains (Fig. 1b through 1d). Primer pair AFF1-AFR3 yielded a specific PCR product of the expected size from all aflatoxigenic strains (Fig. 1e). However, when primer pair AFF1-AFR4 was assayed, nonspecific products were obtained from the 12 tested aflatoxigenic strains (Fig. 1f).

Based on these results, primer pair AFF1-AFR3 was selected to be tested with the 56 reference mold strains. A PCR product of 381 bp was detected from 21 of these strains (Table 1), and the results were in concordance with the data obtained by either MECE or HPLC-MS (Table 1). None of the nonaflatoxigenic reference strains produced a positive PCR result with this primer pair. All aflatoxigenic and nonaflatoxigenic reference mold strains produced a 453-bp amplicon of by PCR with the primer pair Bt2a-Bt2b (based on the β-tubulin gene).

Sensitivity of PCR protocol. The sensitivity of the PCR assay with primer pair AFF1-AFR3 was checked using dilutions of DNA from a pure culture of the aflatoxigenic *A. parasiticus* CBS 571.65. The detection limit was 15 pg of mold DNA (Fig. 2a). However, when DNA from the nonaflatoxigenic *P. viridicatum* CECT 2320 was added to the reaction mixture, the sensitivity of the PCR assay decreased to 25 pg of DNA (Fig. 2b).

The sensitivity of the PCR protocol with primer pair AFF1-AFR3 also was evaluated in various artificially inoculated foodstuffs. After DNA extraction and PCR, the expected amplification product of 381 bp was obtained in all the artificially inoculated food matrices.

No remarkable differences were found between the fungal spore inoculum and the final counts (CFU per gram) obtained by plating. The lowest counts that yielded the expected PCR amplicon ranged from 2.1×10^2 CFU/g in cooked ham to 3.2×10^3 CFU/g in dried fig (Table 3). In general, no remarkable differences were found between the detection limits when the different food matrices were compared (Table 3). Only cured cheese and cooked products had a detection limit significantly lower than those found in the remaining products. In addition, the detection limit was reproducible in the inoculated food products, as indicated by the low variation in the standard deviation usually observed.

PCR inhibition from food components was evaluated by comparing the amplicons obtained using DNA from pure cultures of aflatoxigenic molds mixed with DNA from noninoculated foods with amplicons obtained with DNA

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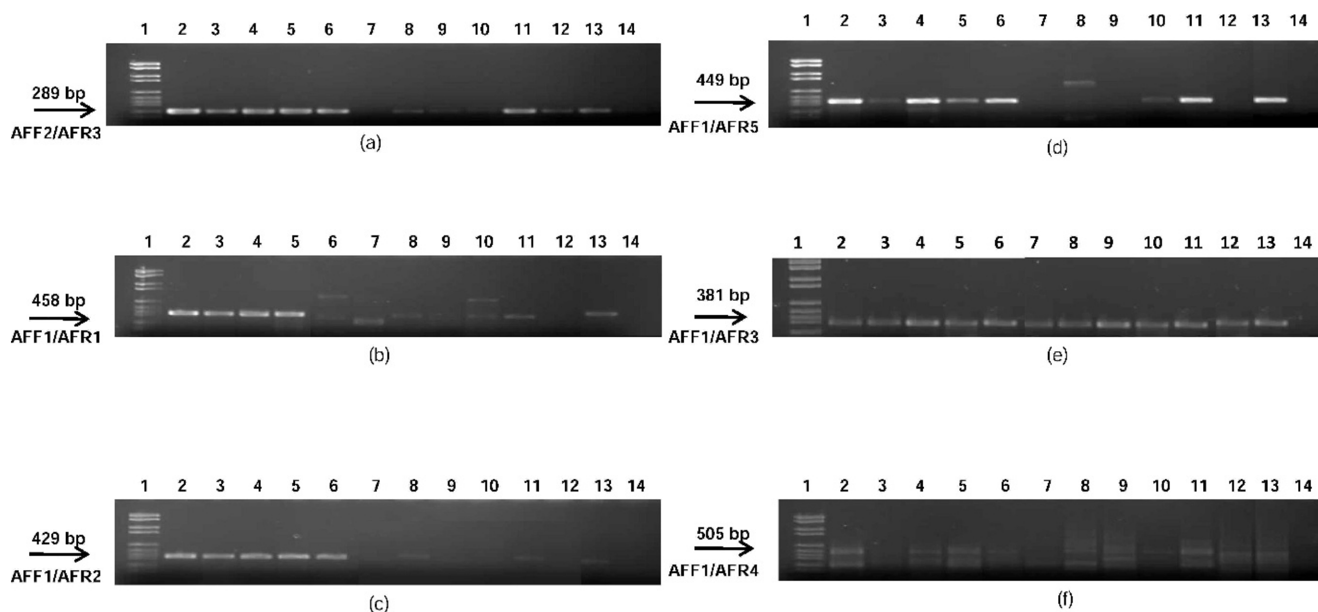

FIGURE 1. *Agarose gel of PCR products obtained with primer pairs AFF2-AFR3 (a), AFF1-AFR1 (b), AFF1-AFR2 (c), AFF1-AFR5 (d), AFF1-AFR3 (e), and AFF1-AFR4 (f) and genomic DNA from* Aspergillus flavus *CECT 2687 (lane 2),* Aspergillus parasiticus *CECT 2688 (lane 3),* A. parasiticus *CECT 2682 (lane 4),* A. flavus *IBT 3696 (lane 5),* A. parasiticus *CECT 2681 (lane 6),* Aspergillus tubingensis *CECT 20545 (lane 7),* A. flavus *CBS 573.65 (lane 8),* A. flavus *CBS 120.62 (lane 9),* Aspergillus ochraceoroseus *CBS 101.887 (lane 10),* A. parasiticus *CBS 571.65 (lane 11),* Aspergillus versicolor *CECT 2814 (lane 12), and* Aspergillus foetidus *CBS 101.708 (lane 13). Lane 1, DNA molecular size marker of 2.1 to 0.15 kbp. Lane 14, negative control using DNA from* Penicillium viridicatum *CECT 2320 as template.*

from pure cultures. Amplification was always obtained with DNA from ripened products, except when 5 µl of DNA from noninoculated samples was added (Fig. 3). PCR amplification of DNA extracted from the remaining foods was always successful. When comparing the spiked amounts of noninoculated matrix DNA, slight differences in the intensity of the amplicons were detected with some of the food products (Fig. 3). No false-positive results were obtained for pure DNA from noninoculated food products.

DISCUSSION

In this work, a PCR protocol able to detect aflatoxigenic molds usually found in foodstuffs was designed. First,

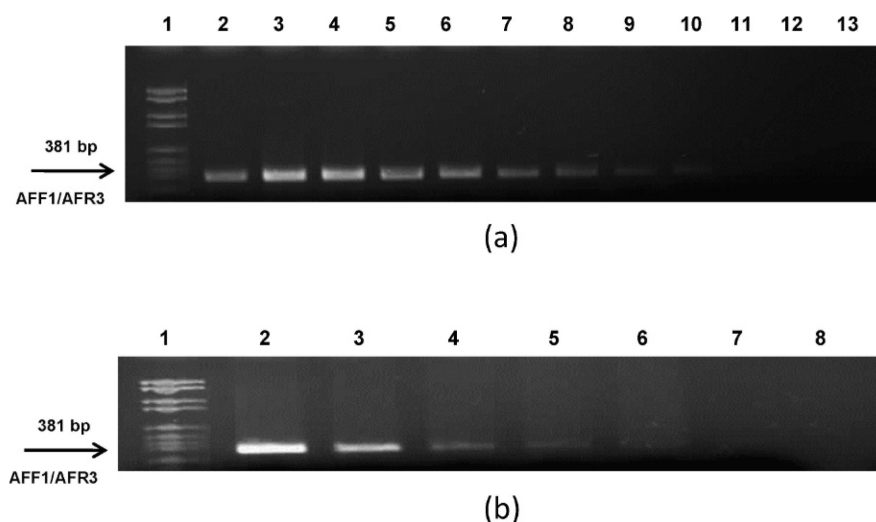

FIGURE 2. *Agarose gels for PCR sensitivity assay with primer pair AFF1-AFR3. (a) PCR products obtained by using decreasing concentrations of DNA from aflatoxigenic* Aspergillus parasiticus *CBS 571.65. Lane 1, DNA molecular size marker of 2.1 to 0.15 kbp; lanes 2 through 12, DNA templates consisting of 50, 25, 15, 5, 0.5, and 0.25 ng and 50, 25, 15, 5, and 0.05 pg of* A. parasiticus *CBS 571.65 DNA; lane 13, negative control using DNA from the nonaflatoxigenic strain* Penicillium viridicatum *CECT 2320. (b) PCR products obtained by mixing a fixed amount of DNA from the nonaflatoxigenic* P. viridicatum *CECT 2320 with decreasing concentrations of* A. parasiticus *CBS 571.65 DNA. Lane 1, DNA molecular size marker of 2.1 to 0.15 kbp; lanes 2 through 7, DNA templates consisting of a mixture of 50 ng of* P. viridicatum *CECT 2320 DNA with 5, 0.5, and 0.05 ng and 25, 15, and 5 pg of* A. parasiticus *CBS 571.65 DNA; lane 8, negative control using DNA of the nonaflatoxigenic strain* P. viridicatum *CECT 2320 as template.*

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TABLE 3. *Detection limits of the PCR method by using the primer pair AFF1-AFR3 and DNA obtained from food products inoculated with aflatoxigenic mold at 10 to 10^6 conidia per g*[a]

Artificially contaminated food	Inoculated mold strains	Inoculum level (conidia/g)[b]	Detected limit (CFU/g)[c]
Cooked products			
Turkey breast	*Aspergillus parasiticus* CECT 2682	$(2.2 \pm 0.26) \times 10^2$ A	$(2.4 \pm 0.31) \times 10^2$ A
Cooked ham		$(2.3 \pm 0.37) \times 10^2$ A	$(2.1 \pm 0.32) \times 10^2$ A
Mortadella		$(2.0 \pm 0.15) \times 10^2$ A	$(2.4 \pm 0.11) \times 10^2$ A
Cured products			
Dry-cured ham	*Penicillium griseofulvum* IBT 14319	$(2.4 \pm 0.48) \times 10^3$ AB	$(2.9 \pm 0.30) \times 10^3$ CD
Dry-fermented sausage (salchichón)		$(2.0 \pm 0.37) \times 10^3$ B	$(2.2 \pm 0.12) \times 10^3$ B
Cured cheese		$(2.2 \pm 0.63) \times 10^3$ AB	$(5.5 \pm 1.15) \times 10^2$ A
Nuts and fruit			
Dried fig	*A. flavus* IBT 3696	$(3.7 \pm 1.45) \times 10^3$ C	$(3.2 \pm 0.20) \times 10^3$ D
Almond		$(2.6 \pm 0.33) \times 10^3$ AB	$(2.7 \pm 0.11) \times 10^3$ BCD
Pistachio		$(2.3 \pm 0.34) \times 10^3$ AB	$(2.5 \pm 0.61) \times 10^3$ BC

[a] Data are the means ± standard deviations of three independent assays. Means followed by different letters are significantly different ($P \leq 0.05$).
[b] Inoculum levels were determined with a Neubauer counting chamber.
[c] Detected limits were obtained after incubating each sample on plates of potato dextrose agar.

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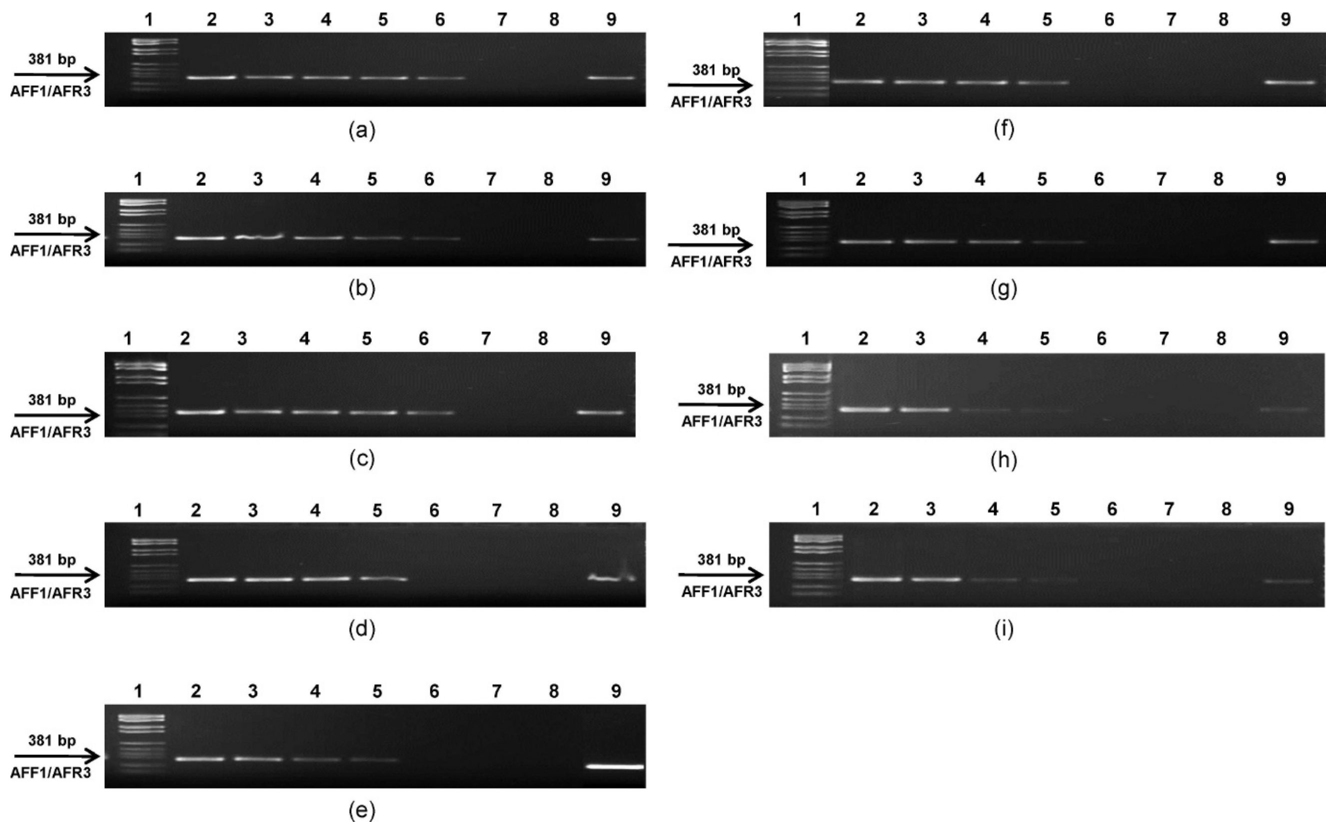

FIGURE 3. *Agarose gels of PCR products obtained by using primer pair AFF1-AFR3 in inhibition assays with food matrices: cooked turkey breast (a), cooked ham (b), mortadella (c), dry-cured ham (d), dry-fermented sausage (salchichón) (e), cured cheese (f), dried fig (g), almond (h), and pistachio (i). Lane 1, molecular weight marker of 2.1 to 0.15 kbp; lanes 2 through 6, DNA template consisting of 5 µl of aflatoxigenic mold DNA from pure cultures mixed with 1, 2, 3, 4, and 5 µl of DNA from each noninoculated food product; lane 7, negative control using sterile deionized water as template; lane 8, DNA template consisting of 5 µl of DNA from noninoculated food samples; lane 9, DNA template consisting of 5 µl of DNA from pure culture of aflatoxigenic strains* Aspergillus parasiticus *CECT 2682 (a through c),* Penicillium griseofulvum *IBT 14319 (d through f), and* Aspergillus flavus *IBT 3696 (g through i).*

the aflatoxin production by 56 reference mold strains was evaluated by MECE and HPLC-MS. These analytical techniques have been described as adequate tools to detect aflatoxin production *(18, 25)*. Twenty-one of the tested strains produced aflatoxins when analyzed by MECE and/or HPLC-MS. For some strains of *Penicillium* (*P. aurantiogriseum* CECT 2264 and *P. griseofulvum* IBT 14319), *Aspergillus* (*A. oryzae* CECT 2094 and CECT 2095, *A. tamarii* CBS 575.65 and CBS 109.63, and *A. tubingensis* CECT 20543 and CECT 20545), and *Rhizopus* (*R. oryzae* CBS 607.68), mycotoxin production was detected by MECE and HPLC-MS even though these strains had never been described as aflatoxin producers. This apparent contradiction could be explained by species misidentifications in the corresponding culture collections. However, after analysis of the partial sequences of the ITS region and β-tubulin gene reported by our research group for these mold strains (GenBank accession nos. JN217227, JN217228, JN217229, JN217230, JN217231, JN217232, JN217233, JN217234, JN217235, JN217236, JN217237, JN217238, JN217239, JN217240, JN217241, JN217242), the strain identifications agreed with those reported by the culture collections in all of the strains except *A. oryzae* CECT 2094 and CECT 2095 and *P. griseofulvum* IBT 14319. *A. oryzae* CECT 2094 and CECT 2095 had greater than 99% similarity to *A. flavus* and *A. parasiticus,* respectively, by both amplification partial sequencing of the ITS region and the β-tubulin gene. These results were in agreement with a more appropriate identification according to production of aflatoxins; *A. flavus* and *A. parasiticus* are mold species usually reported as aflatoxin producers *(30, 42)*. Furthermore, the genetic basis for the inability of *A. oryzae* to produce aflatoxins has been reported *(10, 47)*. Consequently, the identification of both strains as *A. oryzae* by its culture collection should be revised. In the present work, these strains were tentatively renamed as *A. flavus* CECT 2094 and *A. parasiticus* CECT 2095, respectively. With regard to *P. griseofulvum* IBT 14319, its identification did not agree with that reported by its culture collection; it had around 99% similarity to *Penicillium commune*. Thus, this strain was tentatively renamed as *P. commune* IBT 14319. These strains were considered aflatoxigenic and the remaining strains were considered nonaflatoxigenic for the development of the PCR protocol.

The *omt-1* gene was used as the target for the development of a PCR assay for the specific detection of aflatoxigenic molds. The primer pair AFF1-AFR3 was the most appropriate, generating a specific amplicon (381 bp) only in all tested aflatoxigenic strains, when DNA of all aflatoxigenic and nonaflatoxigenic strains were amplified by PCR based on the constitutive β-tubulin gene. These results were very closely related to the detection of aflatoxin production by MECE and HPLC-MS. The *omt-1* gene, which encodes the conversion of sterigmatocystin to *O*-methylsterigmatocystin *(27)* or dihydrosterigmatocystin to dihydro-*O*-methylsterigmatocystin *(49)* depending on the previous expression of the dehydrogenase gene (*aad*), has been reported as a structural gene of the aflatoxin gene cluster together with *nor-1* and *ver-1* and is activated by the *aflR* product *(45)*. Thus, this gene is useful for the detection of aflatoxigenic molds regardless of whether they produce aflatoxins B or G.

The primers OMT-F and OMT-R used for designing the AFF1-AFR3 primer pair were only useful for detecting the two main aflatoxigenic species belonging to the genus *Aspergillus* (*A. flavus* and *A. parasiticus*) *(36)*. These findings were similar to those reported by Manonmani et al. *(28),* who evaluated only a few *Aspergillus* species. However, in the present work the primer pair AFF1-AFR3 was highly sensitive and specific for detection of aflatoxigenic *Aspergillus, Penicillium,* and *Rhizophus* species in pure culture.

Regarding the PCR assay sensitivity, the detection limit in the present study was 15 pg of pure DNA, i.e., lower than the 25 pg reported by others *(15)* as the minimum aflatoxigenic mold DNA concentration that could be detected. However, when the sensitivity of the PCR method was evaluated with pure DNA from an aflatoxigenic strain in the presence of pure DNA from a nonaflatoxigenic mold, a slight decrease in the detection limit was obtained. This effect on the detection limit could be justified by the inhibitory action of the large amount of unspecific DNA over low concentrations of specific mold DNA, apparently by competition *(30)*.

When the potential application of this PCR method for detection of aflatoxigenic molds was evaluated directly with food systems, the detection level ranged between 10^2 and 10^3 CFU/g, depending on the food matrix examined. The lowest detection levels achieved in this study were similar to those found by other authors, who used an enrichment period prior to the extraction of aflatoxigenic mold DNA from the contaminated food matrices *(28, 43, 44)*. In the present study, the PCR assay was able to detect species of *Aspergillus, Penicillium,* and *Rhizopus,* whereas in previous studies the PCR protocols used detected only *A. flavus* and/or *A. parasiticus*.

The small differences in the detection limit between the foods matrices may be caused by inhibitors in the foods, which probably are in higher amounts in those food matrices with higher detection limit (nuts and fruit and cured meat products compared with cooked products and cured cheese). Components of these foods have been reported to inhibit the activity of the *Taq* polymerase used in the PCR assay *(13, 15, 39)*.

To evaluate the influence of food components in PCRs, a last experiment was carried out by parallel amplification of mold DNA from pure cultures mixed with DNA extracted from each noninoculated food. A slight influence of food components on the assay sensitivity was observed. Only the highest amounts of DNA (5 μl) from noninoculated ripened food products seemed to inhibit the PCR. These findings agree with the lowest detection limit of molds obtained in these food matrices (nuts and fruit and ripened foods). Inhibition only with high amount of DNA from foods has also been reported in the detection of aflatoxigenic molds in fresh figs *(15)*.

The PCR protocol developed in this study to detect aflatoxigenic molds is reasonably rapid. It could be performed within 9 h (5 h for treatment and DNA extraction

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from foods and 4 h for analyzing and visualizing PCR results), which is considerably faster than the time needed to detect aflatoxigenic molds using traditional culture methods, including mold identification and subsequent evaluation of aflatoxin production *(23, 24)*.

The PCR assay developed in this study could be used as a routine method to detect aflatoxigenic molds in food manufacturing as part of hazard analysis and critical control point (HACCP) plans, replacing other PCR techniques such as real-time PCR, which requires expensive equipment and consumables and qualified staff. The PCR method developed in this study was used to detect low levels of mold contamination, thus minimizing health risks and other related consumer issues *(34)*. The effective detection of low levels of aflatoxigenic molds would allow the classification of ingredients for either their urgent processing or convenient storage.

In conclusion, the PCR method developed in this study is a rapid, specific, and sensitive tool that can be used to detect aflatoxigenic molds in foodstuffs. This technique may help prevent aflatoxins from entering the food chain because control of colonization by the potentially aflatoxigenic mold could be performed at an early stage of food processing. This PCR protocol could even be used routinely as part of the HACCP systems used by the food industry.

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