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Detection of Aflatoxigenic Molds in Grains by PCR

RONI SHAPIRA,[1]* NACHMAN PASTER,[2] OSNAT EYAL,[1] MAZAL MENASHEROV,[2] ANAIT METT,[1] AND RAFFAEL SALOMON[3]

Institute of Biochemistry, Food Science, and Nutrition, Faculty of Agriculture, The Hebrew University of Jerusalem, Rehovot 76100,[1] and Department of Stored Products[2] and Department of Virology,[3] Agricultural Research Organization (ARO), The Volcani Center, Bet-Dagan 50250, Israel

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Aflatoxins are carcinogenic metabolites produced by several members of the *Aspergillus flavus* group in grains and foods. Three genes, *ver-1*, *omt-1*, and *apa-2*, coding for key enzymes and a regulatory factor in aflatoxin biosynthesis, respectively, have been identified, and their DNA sequences have been published. In the present study, three primer pairs, each complementing the coding portion of one of the genes, were generated. DNA extracted from mycelia of five *Aspergillus* species, four *Penicillium* species, and two *Fusarium* species was used as PCR template for each of the primer pairs. DNA extracted from peanut, corn, and three insect species commonly found in stored grains was also tested. Positive results (DNA amplification) were achieved only with DNA of the aflatoxigenic molds *Aspergillus parasiticus* and *A. flavus* in all three primer pairs. The detection limit of the PCR was determined by using the primer pairs complementing the *omt-1* and *ver-1* genes. Sterile corn flour was inoculated separately with six different molds, each at several spore concentrations. Positive results were obtained only after a 24-h incubation in enriched media, with extracts of corn inoculated with *A. parasiticus* or *A. flavus*, even at the lowest spore concentration applied (10^2 spores per g). No DNA amplification was observed from corn inoculated with other molds, even at the highest inoculum level (10^6 spores per g). It is concluded that genes involved in the aflatoxin biosynthetic pathway may form the basis for an accurate, sensitive, and specific detection system, using PCR, for aflatoxigenic strains in grains and foods.

Aflatoxins are potent carcinogenic, mutagenic, and teratogenic metabolites produced primarily by the fungal species *Aspergillus flavus* and *Aspergillus parasiticus*. Foods and feeds, especially in warm climates, are susceptible to invasion by aflatoxigenic *Aspergillus* species and the subsequent production of aflatoxins during preharvesting, processing, transportation, or storage (7). Over the last few years, means for mycotoxin detection have been simplified. This is mainly because of the official recognition of immunological methods. However, the level of mold infestation and the identification of governing species are still important parameters which could give an indication of the quality of the material as well as of the future potential for the presence of mycotoxins. Mold counts are therefore included in the quality control assurance of many foods. The current methods being used for assessing mold presence are time-consuming, labor-intensive, and costly; require facilities and mycological expertise; and—above all—do not allow the specification of mycotoxigenic strains. Because of the toxic and carcinogenic potential of aflatoxins, there is an urgent need to develop detection methods that are relatively rapid, easily replaceable, and highly specific. PCR facilitates in vitro amplification of a target sequence and offers several advantages over traditional methods of diagnosis: organisms need not be cultured prior to their detection by PCR; the technique possesses exquisite sensitivity, being theoretically capable of detecting target DNA molecules in a complex mixture without the use of radioactive probes; and it is rapid and versatile (1). PCR is now applicable to detection of microorganisms, including plant pathogens (18). However, no information is available to date on the use of PCR for the detection of molds infecting grains and foods, especially mycotoxigenic species.

The generally accepted scheme for aflatoxin biosynthesis is shown in Fig. 1 (reviewed in references 3, 6, and 14). Genes in the aflatoxin biosynthetic pathway were identified by their ability to complement strains of *A. flavus* or *A. parasiticus* having specific blocks in the pathway (20, 26). Using fungal transformation procedures to complement mutations in aflatoxin formation in *A. flavus*, Payne et al. (19) isolated a gene (*afl-2*) which appears to regulate aflatoxin biosynthesis. A homologous gene (*apa-2*) was isolated from *A. parasiticus* (4). Two other genes associated with aflatoxin biosynthesis in *A. parasiticus* were identified by complementation: the *nor-1* gene, associated with the conversion of norsolorinic acid to averantin (5), and the *ver-1* gene, associated with the conversion of versicolorin A to sterigmatocystin (23). Alternatively, purified enzymes involved in aflatoxin biosynthesis have been used to obtain antibodies as immunoscreening probes for cDNA expression libraries. A full-length cDNA clone from *A. parasiticus* (*omt-1*) was isolated, sequenced, and identified as encoding the enzyme involved in the conversion of sterigmatocystin to *O*-methylsterigmatocystin (25).

In this study, we used PCR to selectively distinguish aflatoxigenic molds from other molds and organisms commonly found on grains. We also developed fungal enrichment and DNA extraction techniques for the detection of low numbers of aflatoxigenic molds.

MATERIALS AND METHODS

Organisms and media. The following fungal isolates were used throughout the study: *A. parasiticus* NRRL 5862 (National Center for Agricultural Utilization Research, Peoria, Ill.); *A. flavus* CBS 121.62 (Centraalbureau voor Schimmelcultures, Baarn, The Netherlands); *Aspergillus ochraceus* NRRL 3174; *Aspergillus fumigatus* NRRL 163; *Fusarium moniliforme* NRRL 13616; *Penicillium islandicum* NRRL 1036; and *Fusarium sporotrichioides*, *Fusarium equiseti*, *Aspergillus niger*, *Penicillium expansum*, *Penicillium digitatum*, and *Penicillium italicum* (Department of Stored Products, The Volcani Center, Bet-Dagan, Israel). All fungal cultures were maintained on potato dextrose agar (Difco, Detroit, Mich.). The storage insects *Tribolium castaneum*, *Sitophilus oryzae*, and *Oryzaephilus surinamensis* were from the Department of Stored Products' stock (The Volcani Center).

* Corresponding author. Phone: 972-8-481014. Fax: 972-8-476189.

Fungal spore suspensions (10^6 spores per ml) were obtained by suspending a 7-day-old culture grown on potato dextrose agar in 5 ml of sterile distilled water. Submerged fungal cultures were obtained by inoculating a 250-ml flask containing 75 ml of potato dextrose broth with four agar plaques (1 by 1 cm) excised from the margin of a 3-day culture. Cultures were incubated on a rotary shaker (150 rpm) at 28 ± 1°C for 6 days.

DNA extraction. Total DNA was extracted from 1 g (wet weight) of submerged fungal culture after two cycles of mycelial washing with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) followed by centrifugation. Total DNA was extracted from 1 g of insects or plant material. All biological samples were frozen in liquid nitrogen and pulverized with a mortar and pestle. Lysis buffer (7 ml) (50 mM Tris-HCl [pH 8.0], 150 mM EDTA, 1% [wt/vol] sodium lauroyl sarcosine) was added and mixed gently with the resultant powder, and the mixture was incubated, after addition of 50 μl of RNase cocktail (Ambion, Austin, Tex.), at 37°C for 30 min. Proteinase K solution (1 ml of 2 mg/ml; Boehringer, Mannheim, Germany) was added, and the mixture was incubated for 1 h at 50°C. Double phenol-chloroform extraction was followed by isopropanol precipitation and resuspension in 200 μl of TE buffer (10 mM Tris-HCl, 1.0 mM EDTA [pH 8.0]).

PCR. PCR was used to amplify three target fragments on *A. parasiticus* genomic DNA. Primers VER-496 (5′-ATGTCGGATAATCACCGTTTAGAT GGC-3′, sense strand, bp 496 to 523) and VER-1391 (5′-CGAAAAGCGCCA CCATCCACCCCAATG-3′, antisense strand, bp 1365 to 1391) were used to amplify a 895-bp fragment and were designed on the basis of the published sequence of the *ver-1* gene from *A. parasiticus* NRRL 5862 (23). Primers APA-450 (5′-TATCTCCCCCCGGGCATCTCCCGG-3′, sense strand, bp 450 to 474) and APA-1482 (5′-CCGTCAGACAGCCACTGGACACGG-3′, antisense strand, bp 1458 to 1482) were used to amplify a 1,032-bp fragment and were designed on the basis of the published sequence of the *apa-2* gene from *A. parasiticus* SRRC 2043 (4). Primers OMT-208 (5′-GGCCCGGTTCCTTGGCTCCT AAGC-3′, sense strand, bp 208 to 231) and OMT-1232 (5′-CGCCCCAGTGA GACCCTTCCTCG-3′, antisense strand, bp 1209 to 1232) were used to amplify a 1,024-bp fragment and were designed on the basis of the published sequence of the *omt-1* gene from *A. parasiticus* SRRC 143 (25). Pure submerged cultures of five *Aspergillus* species, four *Penicillium* species, and three *Fusarium* species were lysed, and their genomic DNA was used as template for the PCR. DNA for the PCR was also extracted from peanut, corn, and three insect species commonly found in stored grains.

PCR assays were performed in 50 μl of a reaction mixture that typically contained 100 ng of genomic DNA, deoxynucleoside triphosphates at 200 μM each, primers at 1 μM each, and reaction buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, and 0.2 mg of gelatin per ml). Each reaction mixture was heated to 95°C for 10 min before 2.5 U of *Taq* DNA polymerase (Appligene, Pleasanton, Calif.) was added. A total of 30 PCR cycles (one cycle being 1 min at 94°C for denaturation, 2 min at 65°C for primer annealing, and 2 min at 72°C for extension) and a 5-min final extension at 72°C were run on a programmable DNA thermal cycler (NJ Research Inc., Watertown, Mass.). The PCR products were analyzed by electrophoresis on a 1% agarose gel in 1× TAE (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]) stained with 1 μg of ethidium bromide per ml.

DNA sequence. PCR products generated by each of the three primer pairs were cloned into the TA cloning vector pCRII (Invitrogen, San Diego, Calif.) by standard methods (16). The sequence of 200 nucleotides from each end of the DNA insert was determined by dideoxy sequencing with the Sequenase system (United States Biochemical, Cleveland, Ohio).

Detection of fungal spores in grains. Molds are found in dry stored food and grains mostly as dormant structures, e.g., spores, mycelial fragments, or sclerotia. To test the ability of the PCR to detect aflatoxigenic molds in food, different lots of gamma-irradiated sterilized ground corn (2 g each) were inoculated separately with 10^6 or 10^2 spores per g. The corn was then resuspended in 10 ml of potato dextrose broth and incubated in 50-ml sterile Falcon tubes on an orbital shaker with gentle agitation (50 rpm) at 30°C. Uninoculated corn served as the control. After incubation, the tube contents were centrifuged (5 min at 5,000 × *g*) and the pellets were frozen in liquid nitrogen. From each of the enriched suspension blends, 2-ml portions were taken at 12-h intervals and total DNA was extracted by the above-described procedure. To test the ability of the PCR to distinguish aflatoxigenic molds from other molds found in grains, sterile corn grains were inoculated with 10^6 spores of each of two *Fusarium*, two *Penicillium*, and three nonaflatoxigenic *Aspergillus* species per g.

RESULTS

PCR specificity with *ver-1*, *apa-2*, and *omt-1*. A scheme summarizing the known biochemical steps and genes involved in the aflatoxin biosynthesis is shown in Fig. 1. Amplification products, regardless of the primer set used, were obtained from *A. parasiticus* DNA, and a low-intensity band was seen with *omt-1* and *A. flavus* DNA when 30 PCR cycles were applied (Fig. 2). Each primer pair yielded a single DNA fragment of the expected size: 895, 1,032, and 1,024 bp for *ver-1*, *apa-2*, and *omt-1*, respectively. With 40 PCR cycles instead of 30, a low-intensity band was also observed with the *ver-1* primer set and *A. flavus* DNA. Nonspecific products were never observed, probably because of the relatively high annealing temperature. Cloning and partial sequencing of the PCR products verified each of them to be identical to the original *A. parasiticus* target gene.

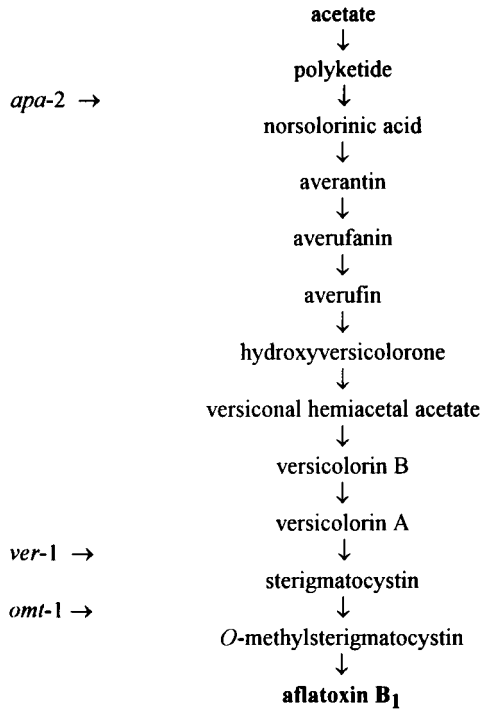

FIG. 1. Schematic illustration of the aflatoxin B_1 biosynthesis pathway. Genes coding for several enzymes are indicated.

PCR studies with inoculated corn. An enrichment procedure in which enriched medium, potato dextrose broth, was added to inoculated ground corn grains which were then incubated was developed. The primer pair generated for the amplification of the *omt-1* gene was tested on aliquots sampled from the enriched cultures after 24 h of incubation. Specific amplification of the *omt-1* gene product was observed only for DNA extracted from corn inoculated with *A. parasiticus* and to a lesser extent with *A. flavus* (Fig. 3). No amplification products were obtained when DNA was extracted from samples without incubation or from inoculated samples with 10^6 spores of two *Fusarium*, two *Penicillium*, and three nonaflatoxigenic *Aspergillus* species per g. Amplification of *ver-1* was observed as a thin band on an agarose gel after as little as 12 h of enrichment with *A. parasiticus*. The band intensity increased gradually when the template DNA was extracted from cultures after 24 and 48 h of enrichment (Fig. 4). The same results were obtained when nonsterile ground corn was inoculated with *A. parasiticus* spores (not shown).

To determine the PCR's sensitivity, lower levels of inoculated spores were tested. By the enrichment procedure, a PCR product generated from the *ver-1* primers was detected, even at the lowest spore level of *A. parasiticus* (10^2 spores per g) after 24 h of enrichment (Fig. 5). No products were observed with the DNA of the uninoculated corn which served as a control. On the basis of the specificity of these results, the PCR products of *ver-1* were cloned into an *Escherichia coli* expression vector to obtain the protein product for future investigations.

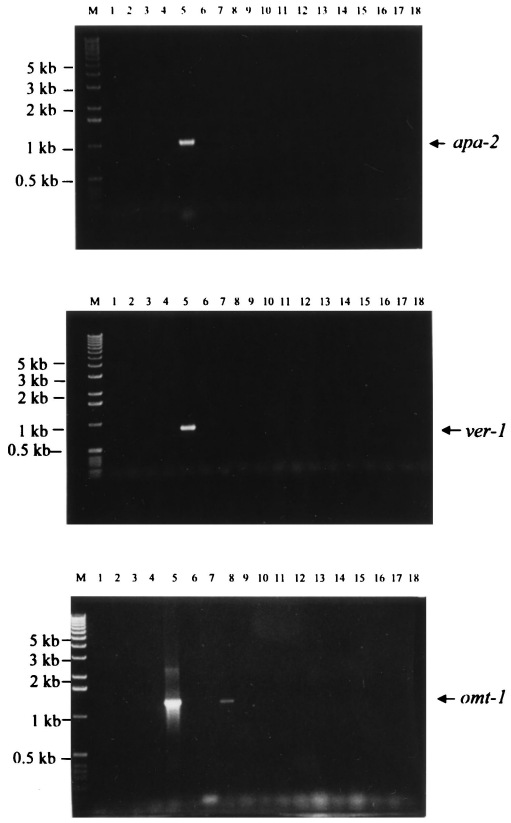

FIG. 2. Gel electrophoretic analysis of PCR products using *apa-2* primers (top), *ver-1* primers (middle), and *omt-1* primers (bottom) of *A. parasiticus*. Lanes: M, DNA molecular size markers; 1, *F. moniliforme*; 2, *F. equiseti*; 3, *F. sporotrichioides*; 4, *A. niger*; 5, *A. parasiticus*; 6, *A. ochraceus*; 7, *A. fumigatus*; 8, *A. flavus*; 9, *P. islandicum*; 10, *P. digitatum*; 11, *P. italicum*; 12, *P. expansum*; 13, *S. oryzae*; 14, *T. castaneum*; 15, *O. surinamensis*; 16, corn; 17, peanut; 18, no DNA.

DISCUSSION

The use of *omt-1* as a probe (25) can discriminate between sterigmatocystin-producing fungi such as some strains of *Aspergillus nidulans* and aflatoxigenic strains. *A. flavus* and *A. parasiticus*, both known to produce aflatoxins, contain *omt-1*. However, *A. nidulans* does not appear to contain a gene homolog of *omt-1*, as indicated by hybridization studies (15). The use of multiple probes, coding for different stages in aflatoxin biosynthesis, should strengthen the validity of a particular strain's identification as aflatoxigenic. Indeed, specific PCR products were obtained only with DNA from the aflatoxigenic mold *A. parasiticus* in all three primer pairs. The difficulty of detecting *A. flavus* due to ambiguous sequences in the primers of the three homolog genes is additional proof of the high sensitivity of our system. The development of selective media for toxigenic molds (8, 21) also fails to confront this issue. DNA probes have revolutionized diagnostic technology in the clinical and forensic fields. Probe-based methods have been developed for the detection and/or enumeration of various foodborne pathogens (12). Recently, a DNA probe containing chromosomal repetitive DNA sequences of *A. flavus* var. *flavus* was constructed and enabled distinction among strains of *A. flavus* belonging to different vegetative compatibility groups (17).

Several recent studies on the use of PCR technology for the detection and diagnosis of fungi have been published (9).

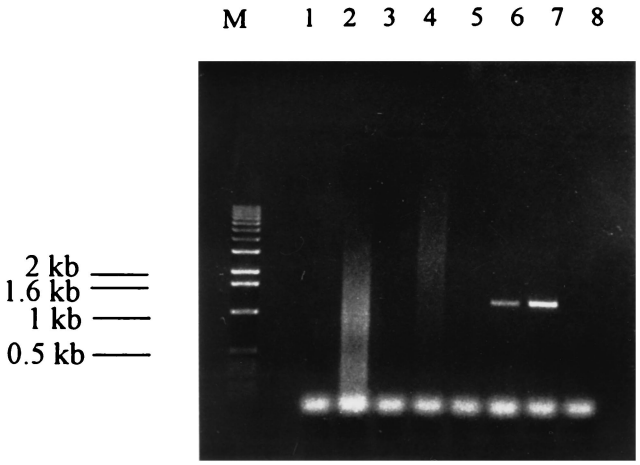

FIG. 3. Gel electrophoretic analysis of PCR products using *omt-1* primers and DNA obtained from extracts of corn grains inoculated with 10^6 spores of the specified mold per g and then incubated under enriching conditions. Lanes: M, DNA molecular size markers; 1, no DNA; 2, *A. ochraceus*; 3, *F. moniliforme*; 4, *P. islandicum*; 5, *F. sporotrichioides*; 6, *A. flavus*; 7, *A. parasiticus*; 8, *P. italicum*.

Quantitation of phytopathogenic fungi in diseased plants is important, especially with the ubiquitous phytopathogens that are present on healthy plants, such as *Verticillium dahliae* and *Verticillium albo-atrum* (10). Likewise, PCR enabled the detection and identification of obligate biotrophic vesicular arbuscular mycorrhizal fungi (24). Nevertheless, the use of PCR to identify specific organisms obtained from a variety of food samples has been problematic because of the presence of various interfering substances (2, 13). Moreover, molds are found on dry food mostly as asexual spores or dried mycelia, which contain only small amounts of DNA and are resistant to cellular disruption for DNA extraction. The detection of *Pseudomonas syringae* pv. phaseolicola from bean seed by PCR was

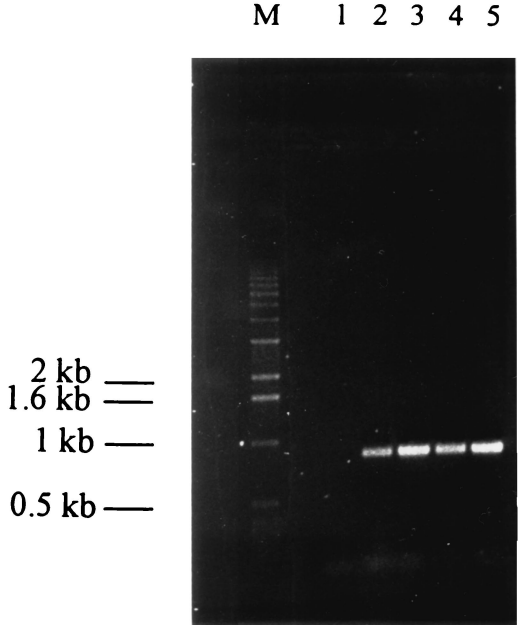

FIG. 4. Gel electrophoretic analysis of PCR products using *ver-1* primers and DNA obtained from extracts of corn grains inoculated with 10^6 spores of *A. parasiticus* per g and then incubated under enrichment conditions. Lane M, DNA molecular size markers. Lanes 1 to 5, template DNA extracted after incubation for 0, 12, 24, 48, and 72 h, respectively.

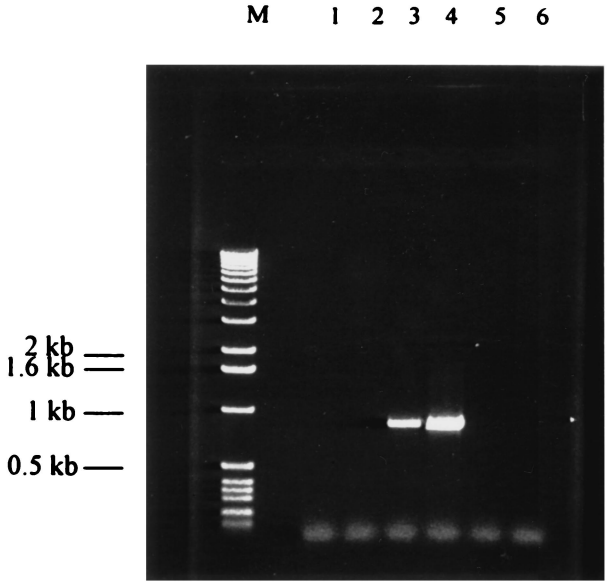

FIG. 5. Gel electrophoretic analysis of PCR products using *ver-1* primers and DNA obtained from extracts of corn grains inoculated with 10^2 spores of *A. parasiticus* per g and then incubated under enriching conditions. Lane M, DNA molecular size markers. Lanes 1 to 4, template DNA extracted after incubation for 0, 12, 24, and 48 h, respectively. Lane 5, template DNA extracted from uninoculated corn grains after 48 h. Lane 6, no DNA.

achieved after incubation with enrichment medium (22). In this study, incubation of dried ground corn seeds in enrichment media (BIO-PCR) allowed us to detect as few as 10^2 spores per g after 48 h of incubation. However, this high sensitivity does not yet represent the detection of a single cell.

In the present study, the novel approach of using selected genes involved in aflatoxin biosynthesis for detecting aflatoxigenic molds is described. Data clearly reveal that the PCR technique is efficient in distinguishing *A. parasiticus* and *A. flavus* from other molds commonly inhabiting stored grains. Current studies are aimed at increasing the detection sensitivity by applying nested primers or immune PCR (1, 11) and at comparing aflatoxigenic and nonaflatoxigenic strains of *A. parasiticus*. The PCR detection limits from parallel lots can then be compared by currently used methods.

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