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ORIGINAL ARTICLE

Polymerase chain reaction-based assay for the early detection of aflatoxigenic fungi on maize kernels

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Keywords

Aspergillus section Flavi; aflatoxin biosynthesis gene cluster; maize; PCR; multiplex PCR.

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Abstract

Objectives The aim of this work was to set up a DNA-based method for detecting aflatoxigenic fungi *Aspergillus flavus* and *Aspergillus parasiticus* on whole maize kernels using species-specific primers. *Methods* In the experiments, two aflatoxigenic strains, *A. flavus* NRRL 3357 and *A. parasiticus* NRRL 2999, were used. Further, *Aspergillus niger* 7096, OTA producer strain, and other strains of *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. isolated from maize kernels were analyzed. Twelve commercial maize hybrids employed in the food and feed industries, not inoculated and inoculated with the different fungi, at different water activities, were used in the polymerase chain reaction-based assay. Three primer pairs were used for amplifying fragments of *Afl P* (*omt 1*), *Afl M* (*ver 1*), *Afl R*, genes present in the aflatoxin biosynthesis cluster. *Results* DNA amplification was achieved only with DNA from fungal strains of *A. parasiticus* and *A. flavus* and from maize kernels inoculated with *A. flavus* or *A. parasiticus*. Amplification was never observed with DNA of the other fungal species. Amplification was evident in maize starting from 12 h of incubation after inoculation, when mycelium is not yet visible by stereomicroscope analysis. *Conclusion* The results indicate that these highly specific and sensitive polymerase chain reaction-based methods are able to discriminate maize kernels infected with *A. flavus* or *A. parasiticus* from uninfected ones. This polymerase chain reaction method could be a real, effective alternative to traditional diagnostic methods for the early detection of aflatoxigenic fungi in food commodities.

Introduction

Fungi can grow on many food commodities. Some fungal strains such as *Aspergillus flavus* and *Aspergillus parasiticus* can produce, under suitable conditions, aflatoxins and secondary metabolites which are toxic for human and animals (Haouet & Altissimi, 2003; Fanelli *et al*., 2004).

Aflatoxins (AF) are hepatocarcinogenic compounds, synthesized through a complex enzymatic pathway (Yu *et al*., 2004). The main aflatoxins are AF B1, B2, G1, G2, and M1, a metabolic thermostable derivative of AF-B1, which is accumulated in the milk of cows fed with aflatoxin-contaminated feed. Not all of the strains of *A. flavus* and *A. parasiticus* are aflatoxigenic: in fact, many isolates are nontoxigenic due to mutations in one or more genes of the biosynthetic gene cluster (Criseo *et al*., 2001>; Ehrlich and Cotty, 2004).

Aflatoxigenic fungi represent a real issue, especially for the cereal industry. These fungi usually colonize cereals (maize, wheat, barley) in postharvest conditions. Contamination by aflatoxigenic fungi is a significant problem for maize, a cereal employed, above all, in feed composition, harvested with a moisture content of about 18–20% (0.90–0.93 a.w.) and then dried. Environmental conditions can often determine fungal spoilage and aflatoxin production if the drying process is incorrect. Aflatoxin

contamination may be prevented by the early identification of the toxigenic fungi directly into the feed- and foodstuffs. In fact feed and food producers can easily recognize using real-time methodologies the difference between contaminated stuffs and uncontaminated ones.

Many methods have been utilized for measuring fungal contamination and the presence of toxins in and on food. Traditional methods for identifying aflatoxigenic fungi (microbiological methods and diagnostic media) or immunological methods for toxin detection are generally time consuming or not fully reliable. Several research groups have analyzed the possibility for rapid and early detection of aflatoxigenic fungi in food commodities, using polymerase chain reaction (PCR)-based methods, which are able to diagnose for either the presence or the expression of aflatoxins' biosynthetic gene cluster (Shapira *et al.*, 1996).

The aim of this work was to use a PCR-based method to detect aflatoxigenic fungi on maize using species-specific primer pairs designed in the aflatoxin gene cluster. These genes should be present only in aflatoxin producing fungi, therefore they could represent sensitive and specific markers for the early detection of aflatoxigenic strains in food and feed commodities. The multiplex PCR reaction for detecting aflatoxigenic fungal strains was tested too, amplifying three target genes in the same PCR reaction mix, to differentiate with a higher degree of effectiveness between potentially aflatoxigenic and nonaflatoxigenic strains. Multiplex PCR, based on genes involved in different phases of aflatoxins' biosynthesis, can be useful in confirming the identification of a potentially aflatoxigenic specie.

Materials and methods

Fungal strains and culture conditions

Fungal strains were supplied by the fungal collections of Prof. C. Fanelli at University of Rome 'Sapienza'. In the experiments two aflatoxigenic strains, *A. parasiticus* NRRL 2999 and *A. flavus* NRRL 3357, were used to develop the method. *Aspergillus niger* 7096 strain, and other strains of *Aspergillus ochraceus*, *Penicillium verrucosum*, and *Fusarium graminearum* isolated from maize kernels were also used to test the specificity of PCR method presented.

Maize

In the PCR-based assay 12 commercial maize (*Zea mays* L.) hybrids (see Table 1) supplied by the Istituto Sperimentale di Cerealicoltura (Rome, Italy) were used. One hybrid (Cecilia) was used to develop the method, the others to test the method on different samples not artificially contaminated. The aliquot (20 g) of maize used for artificial inoculation with single or mixed fungal strains, was previously remoistened to 30% by adding distilled sterile water at 30 °C.

DNA extraction and amplification

Genomic DNA was extracted from 30 mg (dry weight) of freeze-dried fungal mycelia of *Aspergillus*, *Penicillium*, and *Fusarium* species harvested from freshly growing cultures in potato dextrose broth and from uninoculated and inoculated freeze-dried maize kernels. DNA extraction was performed according to the Farber method (Farber *et al.*, 1997) slightly modified; in our extraction protocol we used a mixture of phenol–chloroform–isoamyl alcohol (25:24:1) instead of pure phenol for the purification of the sample and isopropanol instead of absolute ethanol to precipitate DNA. The precipitate DNA was washed with 70% ethanol and finally resuspended in 50 µL of filter-sterilized distilled water.

Two primer pairs employed in previous works, designed on the coding sequence of genes *afl P* and *afl M*: OMT-208

Table 1 Maize hybrids used in experimental assays

Maize hybrids	FAO class	Activity water (a.w.)
DK 537	400	0.79
LATINA	600	0.64
DKC 6843	700	0.67
COSTANZA	600	0.66
CECILIA	500	0.65
DKC 5143	400	0.67
ARSANO	300	0.73
CORNIOLA	300	0.72
SIV 6450	500	0.85
DUENDE	600	0.77
NK CRISO	500	0.84
MITIC	600	0.87

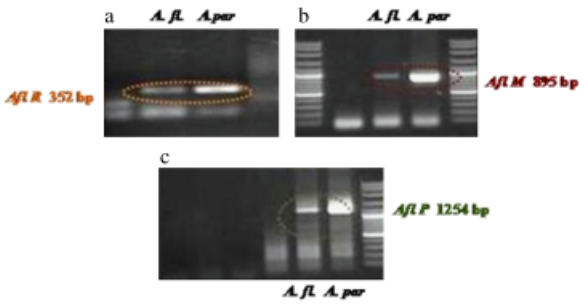

Figure 1 Single polymerase chain reaction amplification, with the primer pairs for *Afl R* (a), *Afl M* (b), and *Afl P* (c), of *Aspergillus flavus* 3357 and *Aspergillus parasiticus* 2999 genomic DNA.

(5′-GGCCCGGTTCCTTGGCTCCTAAGC-3′ and OMT-1232 5′-GCCCCAGTGAGACCCTTCCTCG-3′) (Shapira *et al*., 1996), VER-496 (5′-ATGTCGGATAATCACCGTTTA GATGGC-3′ and VER-1391 5′-CGAAAAGCGCCACCATC CACCCCAATG-3′), (Shapira *et al*., 1996) were used. For the *afl R* gene, a primer pair AFLR-forward (5′-AATACAT GGTCTCCAAGCGG-3′) and AFLR-reverse (5′-GAAGACA GGGTGCTTTGCTC-3′) was designed with the Primer 3 software (Whitehead_Institute and Howard Hughes Medical Institute designed by Steve Rozen; http://frodo.wi.mit.edu/primer3/) on the coding sequence of *Afl R* gene.

PCR amplifications produced fragments of 1254 bp length for *Afl P*, 895 bp for *Afl M*, and 352 bp for *Afl R*, specific for *A. flavus* and *A. parasiticus*, potential aflatoxin producers. DNA amplification conditions were as follows: 3 min initial step, followed by 37 cycles at 94 °C for 1 min, 66 °C for 2 min, 72 °C for 2 min, and a final extension step at 72 °C for 8 min. Amplification operative conditions were optimized on genomic DNA from pure fungal strains and confirmed on DNA extracted from maize kernels. In multiplex PCR all the target genes fragments (*afl R*, *afl M*, and *afl P*) were amplified in the same PCR reaction mix, at the same operative conditions of single PCR for *Afl P* and *Afl M*.

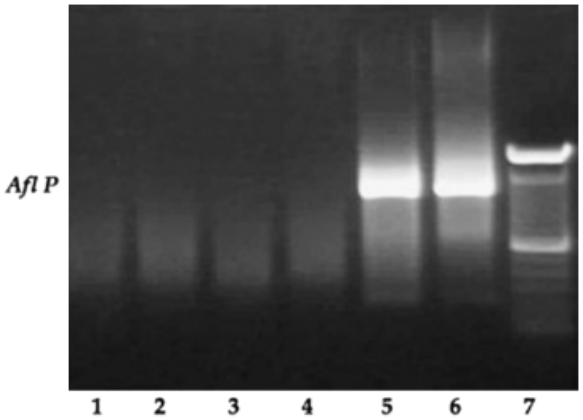

Figure 2 Single polymerase chain reaction (PCR) amplification with the primer pairs for *Afl P* of genomic DNA from mixed commercial fungal strains and naturally occurring fungal contaminants isolated from maize kernels. Lane 1: PCR-negative Control, lane 2: DNA from nonaflatoxigenic mixed fungal strains (*Rhizopus arrhizus*, *Aspergillus niger*, *Penicillium verrucosum*), lane 3: DNA from nonaflatoxigenic mixed fungal strains (*Fusarium graminearum*, *Aspergillus niger*, *Penicillium verrucosum*), lane 4: DNA from nonaflatoxigenic mixed fungal strains (*Rhizopus arrhizus*, *Aspergillus ochraceus*, *Penicillium verrucosum*, *Fusarium graminearum*); lane 5: mixed fungal strains (the same as lane 4) with aflatoxigenic *Aspergillus flavus* 3357; lane 6: mixed fungal strains (the same as lane 4) with aflatoxigenic *Aspergillus parasiticus* 2999, lane 7: DNA marker.

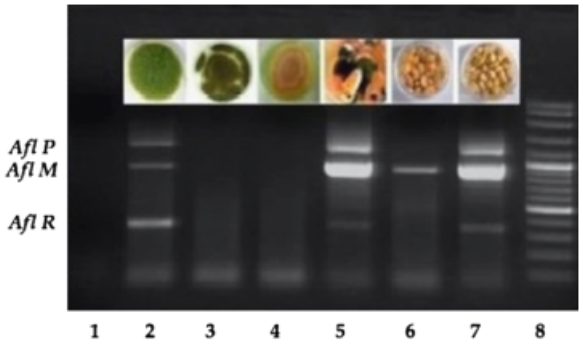

Figure 3 Multiplex polymerase chain reaction (PCR) amplification with the primer pairs for *Afl R* (352 bp), *Afl M* (895 bp), and *Afl P* (1254 bp), of genomic DNA from different fungal strains and maize kernels. Lane 1: PCR-negative control, lane 2: *Aspergillus flavus* 3357; lane 3: *Aspergillus. niger*, lane 4: *Fusarium graminearum*, lane 5: mixed Mycelium (*A. niger*, *A. flavus*, *Penicillium verrucosum*, and *Rhizopus arrhizus*) isolated from maize kernels, lane 6: uninoculated maize kernels, lane 7: maize kernels inoculated with *A flavus* 3357, lane 8: DNA marker.

Table 2 Fungal species used for testing primer pairs specificity

	Gene target		
Fungal species	*Afl R*	*Afl M*	*Afl P*
Aspergillus ochraceus	–	–	–
Aspergillus niger 7096	–	–	–
Fusarium graminearum	–	–	–
Rhizopus arrhizus	–	–	–
Fungal mixtures without aflatoxigenic strains[1]	–	–	–
Fungal mixtures with aflatoxigenic strains[2]	+	+	+

[1]*Aspergillus ochraceus*, *Rhizopus arrhizus*, *Fusarium graminearum*, and *Penicillium viridicosum*.
[2]*Aspergillus ochraceus*, *Rhizopus arrhizus*, *Fusarium graminearum*, *Penicillium viridicosum* and aflatoxigenic *Aspergillus flavus* 3357.

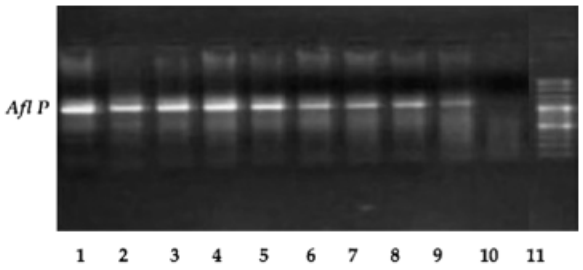

Figure 4 Single polymerase chain reaction (PCR) amplification with the primer pair for *Afl P* of different concentrations of DNA (10 pg–100 ng) from *Aspergillus flavus* 3357; lane 1: 100 ng, lane 2: 50 ng, lane 3: 25 ng, lane 4: 10 ng; lane 5: 5 ng, lane 6: 1 ng, lane 7: 0.5 ng, lane 8: 0.25 ng, lane 9: 0.01 ng, lane 10: PCR-negative control, lane 11: DNA marker.

Sensitivity and effectiveness of PCR reaction method

To determinate the sensitivity of the method a range of *A. flavus* 3357 genomic DNA concentrations (5 pg–100 ng) were amplified in single and multiplex PCR. The same was carried out using the aflatoxigenic strain *A. parasiticus* 2999. In order to test whether PCR assay could be affected by matrix DNA, the sensitivity of the primers was also tested in the presence of 500 pg of DNA from maize virtually free of fungal *A. flavus* or *A. parasiticus* infection.

Detection of aflatoxigenic fungi on maize kernels

The sensitivity and the effectiveness of PCR for detecting aflatoxigenic fungi on maize kernels, were tested on different lots of superficially sterilized whole maize kernels (cv. Cecilia) humidified at 30% m.c.s The sterilization was performed by adding to the maize kernels a sterilizing solution of 2.5% v/v Clorex (NaClO), under agitation for 20 min, and then washing three times with sterile distilled water. The effectiveness of the surface sterilization procedure was determined by placing maize kernels (five kernels per plate for a total of 20 plates) in Petri plates and monitoring the possible fungal development over a 7-day period of incubation under stationery conditions at 30 °C. Maize kernels (20 g per flask) were artificially contaminated with *A. flavus* 3357 or *A. parasiticus* 2999 by adding 2 mL of 2×10^6 conidia suspension harvested from a pure culture of *A. flavus* 3357 or *A. parasiticus* 2999 (10^4 conidia g^{-1} maize kernels). After mixing by manually shaking to enhance uniformity, the maize samples were incubated at 30 °C under stationery conditions. Total DNA was then extracted by the procedure described above at 12-h time intervals (time 0, 12, 24, 36, 48, and 72 h). Two independent experiments were performed; for each experiment three replicates (i.e. flasks) per time interval were carried on.

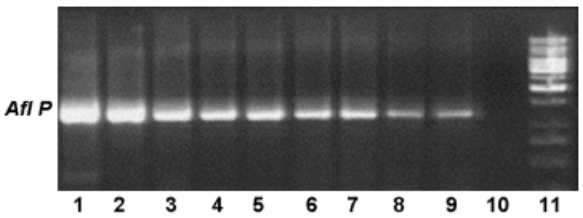

Figure 5 Single polymerase chain reaction (PCR) amplification with the primer pair for *Afl P* of different concentrations of DNA (10 pg–100 ng) from *Aspergillus parasiticus* 2999; lane 1: 100 ng, lane 2: 50 ng, lane 3: 25 ng, lane 4: 10 ng; lane 5: 5 ng, lane 6: 1 ng, lane 7: 0.5 ng, lane 8: 0.25 ng, lane 9: 0.01 ng, lane 10: PCR-negative control, lane 11: DNA marker.

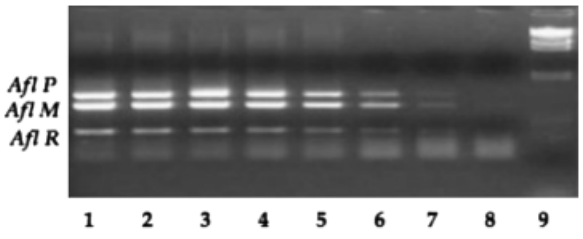

Figure 6 Multiplex polymerase chain reaction (PCR) amplification with the primer pairs for *Afl R*, *Afl M*, and *Afl P*, of different concentrations of DNA (500 pg–100 ng) from *Aspergillus flavus* 3357; lane 1: 100 ng, lane 2: 50 ng, lane 3: 25 ng, lane 4: 10 ng; lane 5: 5 ng, lane 6: 1 ng, lane 7: 0.5 ng, lane 8: PCR-negative Control, lane 9: DNA marker.

Results and discussions

The PCR assay for fungal detection developed in this study, based on the amplification of genes involved in the biosynthesis of the aflatoxins, was able to detect the most

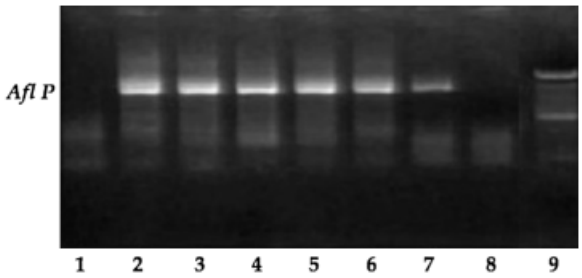

Figure 7 Single polymerase chain reaction (PCR) amplification with the primer pair for *Afl P* of different concentrations of *Aspergillus flavus* 3357 genomic DNA (50 pg–100 ng), spiked with 500 pg of DNA from maize virtually free of fungal *A. flavus* or *Aspergillus parasiticus* infection. Lane 1: PCR-negative control, lane 2: 100 ng, lane 3: 50 ng, lane 4: 25 ng, lane 5: 10 ng.; lane 7: 1 ng, lane 7: 0.1 ng, lane 8: 0.05 ng, lane 9: DNA marker.

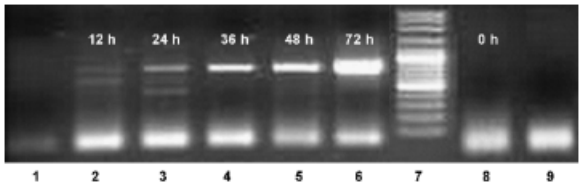

Figure 8 Single polymerase chain reaction (PCR) amplification with the primer pair for *Afl M* of genomic total DNA extracted from superficially sterilized maize kernels (20 g) inoculated with *Aspergillus flavus* (10^4 conidia g^{-1} of maize) after 0, 12, 24, 36, 48, and 72 h of incubation at 30 °C. Lane 1: PCR-negative control, lane 2: maize+*A. flavus* 12 h, lane 3: maize+*A. flavus* 24 h, lane 4: maize+*A. flavus* 36 h, lane 5: maize+*A. flavus* 48 h, lane 6: maize+*A. flavus* 72 h, lane 7: DNA marker, lane 8: maize+*A. flavus* 0 h, lane 9: uninoculated maize.

Table 3 Testing of primer pairs on different commercial maize hybrids

	Gene target		
Maize hybrids	*Afl R*	*Afl M*	*Afl P*
DK 537	+	+	+
LATINA	+	+	+
DKC 6843	−	−	−
COSTANZA	−	−	−
CECILIA	−	−	−
DKC 5143	−	−	−
ARSANO	−	−	−
CORNIOLA	−	−	−
SIV 6450	+	+	+
DUENDE	+	+	+
NK CRISO	+	+	+
MITIC	+	+	+

important aflatoxin-producing species *Aspergillus* Section Flavi (*A. flavus* and *A. parasiticus*).

The specificity of selected primer pairs for detecting only potential aflatoxigenic fungal strains and not other natural contaminants of maize, was confirmed with DNA extracted from different aflatoxigenic and nonaflatoxigenic fungal strains and with DNA extracted from artificially inoculated maize. Positive amplifications were achieved only with DNA from *A. flavus* 3357 and *A. parasiticus* 2999 fungal strains and from maize inoculated with *A. flavus* 3357 or *A. parasiticus* 2999 (Figure 1) (Figure 2 lanes 5 and 6) (Table 2), but never with DNA from other fungal strains (Table 2) (Figure 2 lanes 2– 4). The specificity of the amplification's protocol has also been confirmed in Multiplex PCR reactions, in which all the primer pairs have been used in a single mix of amplification.

Multiplex PCR showed that only *Aspergillus* sez. Flavi fungal strains (Figure 3, lanes 2, 5, and 7) gave a triplet pattern, indicating the presence of the three genes of the aflatoxin biosynthetic pathway selected. The presence of a complete pattern shows that it could be a sufficient marker to detect potentially aflatoxinogenic strains even if further studies should be carried out in order to confirm these preliminary data.

The proposed PCR amplification method described herein was particularly sensitive. The estimated detection limit *in vitro* was 10 pg of DNA template in the single PCR for all the genes tested [e.g. *Afl P* for *A. flavus* 3357 (Figure 4) and *A. parasiticus* 2999 (Figure 5)]. As expected, the sensitivity of the multiplex PCR method was lower than single PCR method, with an estimated detection threshold of 500 pg of DNA template for *A. flavus* 3357 (Figure 6) and *A. parasiticus* 2999 (data not shown). The execution of protocols of Multiplex PCR, based on genes involved in different phases of aflatoxin biosynthesis, can, however, result useful for confirming the identification of a potentially aflatoxigenic species.

Because the main objective of this work was to develop a protocol to be used directly in feed and food commodities, sensitivity, and robustness on real matrix are critical factors. These PCR-based assays had shown high sensitivity and effectiveness, detecting a quantity of genomic DNA estimated in 100 pg g^{-1} of maize kernels for *A. flavus* 3357 (Figure 7) and *A. parasiticus* 2999 (data not shown).

Moreover, the amplification of fungal DNA extracted from maize kernels artificially inoculated with *A. flavus* 3357 or *A. parasiticus* 2999 conidial suspension (10^4 conidia g^{-1} maize kernels) in the different time intervals (time 0, 12, 24, 36, 48, and 72 h) of incubation at 30 °C for 6 days, has shown an increase of amplification signal up to 72 h, starting at 12 h after inoculation, when mycelium is not visible to stereomicroscope (100 ×) observation. In Figure 8 the amplification profile of *A. flavus* 3357 is shown for different time intervals of incubation (0–72 h) on maize kernels. A similar amplification profile was obtained for *A. parasiticus* 2999 inoculated on maize kernels for the same time intervals (data not shown). Therefore, this system has proved effective for the early detection of aflatoxigenic contaminants into the maize kernels.

Another amplification protocol, which used the *Afl P* and *Afl M* primers has been developed from Shapira *et al*. (1996). This study describes a method characterized by an amplification sensitivity similar to that presented in this paper. Nevertheless, our study represents a significant improvement of the method as we obtained amplification using simultaneously three primer pairs such as *Afl P*, *Afl M*, and *Afl R* in a multiplex PCR assay. Moreover, the method here described allows us to extract and amplify DNA directly from the contaminated kernels (both by *A. flavus* and *A. parasitcus*), i.e. with no need for further incubation and mycelia enrichment using culture media. Thus, our method minimizes the time procedures and optimizes the amplification protocol respect to previously presented assays.

Furthermore, some positive amplifications were obtained when kernels of different commercially available maize hybrids, not artificially contaminated, were used in this work (Table 3). In particular, the DNA extracted from maize hybrids DK 537, DUENDE, SIV 6450, MITIC, and NK CRISO, was positively amplified using *Afl P*, *Afl M*, and *Afl R* primer pairs. The kernels of these hybrids were then incubated for 7 days at 30 °C and five strains of aflatoxigenic

fungi were isolated in pure culture and recognized as *A. flavus*, both by traditional and molecular tools.

The PCR method discussed above could be a real and effective alternative compared with the traditional diagnostic methods for the early detection of aflatoxigenic fungi in naturally contaminated food commodities.

The method proposed in this work represents a useful tool for evaluating the quality of raw materials at different critical points in the food chain. It could be used to predict the potential risk for the presence of potentially aflatoxigenic strains. The combination of this approach with the more expensive and laborious conventional chemical analysis of toxins would improve the control of toxins entering the cereal food chain.

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