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## Highly sensitive PCR-based detection method specific to Aspergillus flavus in wheat flour

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Belén Patiño

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**Highly sensitive PCR-based detection method specific to  
Aspergillus flavus in wheat flour**

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3 **Highly sensitive PCR-based detection method specific to *Aspergillus***  
4 ***flavus* in wheat flour**  
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11 **Abstract**  
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13 *Aspergillus flavus* is an important fungal species which frequently occurs in food  
14 commodities producing a wide array of toxins, aflatoxins being the most relevant in  
15 food safety. In this work, a PCR-based protocol is described specific to this species  
16 which allowed discrimination from other closely related species having different  
17 profiles of secondary metabolites from the *Aspergillus* Section *Flavi*, particularly *A.*  
18 *parasiticus*. The specific primers were designed on the multi-copy internal transcribed  
19 region of the rDNA unit (ITS1-5.8S-ITS2 rDNA) and were tested in a wide sample of  
20 related species and other fungal species commonly found in commodities. The PCR  
21 assay was coupled with a fungal enrichment and a DNA extraction method for wheat  
22 flour to enhance the sensitivity of the diagnostic protocol. The results indicated that the  
23 critical PCR amplification product was clearly observed for wheat flour contaminated  
24 by 10<sup>2</sup> spores after 16 h of incubation.  
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44 **Keywords:** *Aspergillus flavus*, *aflatoxin*, *polymerase chain reaction (PCR)*, *detection*,  
45 *wheat*, *flour*, *internal transcribed spacer (ITS)*  
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51 **Running head** PCR detection method for *Aspergillus flavus* in wheat flour  
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## Introduction

*Aspergillus* Section *Flavi* contains a number of species capable of producing a wide array of toxins among which aflatoxins are the most important in food safety. Aflatoxins are potent carcinogenic, mutagenic, and teratogenic secondary metabolites and are produced predominantly by *Aspergillus flavus* and *Aspergillus parasiticus* (Bennett and Papa, 1988). This Section consists of two groups of species; one includes *Aspergillus flavus*, *A. parasiticus*, *A. nomius* and the recently described species *A. pseudotamarii* and *A. bombycis* (Cary and Ehrlich 2006; Ehrlich et al. 2006), all of which produce aflatoxin. The other group includes the aflatoxin non-producing species *A. oryzae*, *A. sojae*, and *A. tamarii*, which have been used for production of traditional fermented foods in Asia (Kumeda and Asao 2001).

Aflatoxinogenic fungi can contaminate several food commodities including cereals (Pittet 1988), peanuts (Jelinek et al. 1989), spices (Bartine and Tantaoui-Elaraki 1997) and figs (Doster et al. 1996; Färber et al. 1997). Foods and feeds are especially susceptible of colonization by aflatoxinogenic *Aspergillus* species in warm climates where they may produce aflatoxins along several stages of the food chain: either at pre-harvest, processing, transportation or storage (Ellis et al. 1991). 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 (Shapira et al. 1996).

The traditional methods for identification and detection of these fungi in foods include culture in different media and morphological studies. This approach, however, is very

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3 time-consuming, laborious, and requires facilities and mycological expertise (Edwards  
4 et al. 2002). Moreover, these methods have low degree of sensitivity and do not allow  
5 the specification of mycotoxigenic species (Zhao et al. 2001). PCR-based methods that  
6 target DNA are considered a good alternative for rapid diagnosis because of their high  
7 specificity and sensitivity, and have been used for the detection of aflatoxigenic strains  
8 of *A. flavus* and *A. parasiticus* (Shapira et al. 1996; Färber et al. 1997; Sweeney et al.  
9 2000; Criseo et al. 2001; Chen et al. 2002; Mayer et al. 2003; Zachová et al. 2003;  
10 Somashekar et al. 2004). However, none of these methods has yet been able to reliably  
11 differentiate *A. flavus* from other species of the *A. flavus* group. In particular, *A. flavus*  
12 and *A. parasiticus* have two different toxicogenic profiles. *A. flavus* produces aflatoxin  
13 B1 (M1) B2, cyclopiazonic acid, aflatrem, 3-nitropropionic acid, sterigmatocystin,  
14 versicolorin A and aspertoxin, whereas *A. parasiticus* produced aflatoxin B1 (M1), B2,  
15 G1, G2 and versicolorin A (Wilson et al. 2002). Another important fact is that  
16 *Aspergillus flavus* and *A. fumigatus* are responsible for 90 % of the aspergillosis in  
17 human beings (Paya 1993).

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41 Most of the PCR protocols are based on aflatoxin biosynthetic genes which are single  
42 copy and, therefore, less sensitive than multi-copy genes (Bluhm et al. 2002, Jurado et  
43 al. 2006). The intergenic spacer (IGS) and the internal transcribed region (ITS) from the  
44 rDNA units are multicopy (up to 100 or 300 copies per haploid fungal genome) and  
45 highly variable sequences which are widely used for phylogenetic studies and  
46 diagnostics of closely related fungal species such as *Aspergillus* (Henry et al. 2000;  
47 Parenicová et al. 2001; Zhao et al. 2001; Varga et al. 2004; González-Salgado et al.  
48 2005; Patiño et al. 2005) or *Fusarium* (González-Jaén et al. 2004; Mirete et al. 2004;  
49 Patiño et al. 2004).

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6 In this study, we have developed a PCR protocol based on multi-copy sequences (ITS)  
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8 specific to *A. flavus* which allows distinction from other aflatoxinogenic molds, in  
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10 particular from *A. parasiticus*, and organisms commonly found on grains. The method  
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12 proved to be very efficient even at short incubation times and at low spores  
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14 concentration of *A. flavus* in wheat flour samples.  
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## 17 18 19 20 **Materials and methods**

### 21 22 *Fungal isolates and culture conditions*

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24 A detailed description of the fungal strains used in this study is given in Table I. All  
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26 fungal cultures were maintained on potato dextrose-agar (PDA, Scharlau Chemie,  
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28 Barcelona, Spain) at 4°C and stored as spore suspension in 15% glycerol at –80°C. The  
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30 isolates were cultured in 100 mL Erlenmeyer flasks containing 20 mL liquid medium  
31  
32 Sabouraud (Scharlau Chemie, Barcelona, Spain). Cultures were inoculated with  
33  
34 mycelial disks cut from the plates and incubated at 25°C, 150 rpm. Mycelia from 2-day-  
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36 old cultures were harvested by filtration through Whatman paper n° 1 and kept at –80°C  
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38 for DNA isolation.  
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42 [Insert Table I]  
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### 48 49 *DNA extraction and PCR amplification*

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51 Genomic DNA of the strains was obtained using either the genomic DNA Extraction  
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53 Kit (Genomix, Talent, Trieste, Italy), according to the manufacturer's instructions. All  
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55 genomic DNAs used in this work were tested for suitability for PCR amplification using  
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57 primers ITS1 and ITS4 (Henry et al. 2000), which amplify the ITS region in  
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59 *Aspergillus*. The PCR reaction was performed in an Eppendorf Mastercycler Gradient  
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3 (Eppendorf, Hamburg, Germany) using 10 pg-10 ng of genomic DNA. The ITS1-5.8S-  
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5 ITS2 sequences were obtained following the same protocol by Henry et al (2000) above  
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7 mentioned and the PCR amplification products obtained and purify using the High Pure  
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9 PCR Product purification Kit (Roche, Germany) according to the manufacturer's  
10  
11 instructions. The PCR products were subsequently sequenced by both strands using the  
12  
13 ABI PRISM DNA Sequencer (Applied Biosystems, Foster City, USA) according to the  
14  
15 manufacturer's instructions in the Genomic Unit of the Complutense University of  
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17 Madrid. These sequences and those retrieved from GenBank and other data bases were  
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19 analysed and aligned by Clustal method using the program DNASTAR (Lasergene,  
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21 Wisconsin, USA).

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29 Specific PCR assays were carried out using primers FLA1 (5'  
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31 GTAGGGTTCCTAGCGAGCC 3') and FLA2 (5' GGAAAAAGATTGATTTGCG  
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33 TTC 3') for *A. flavus*. PCR reactions were performed in an Eppendorf Mastercycler  
34  
35 Gradient (Eppendorf, Hamburg, Germany). The PCR amplification protocol used for *A.*  
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37 *flavus* was as follows: 1 cycle of 5 min at 95°C, 26 cycles of 30s at 95°C  
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39 (denaturalization), 30s at 58°C (annealing), 45 s at 72°C (extension) and finally 1 cycle  
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41 of 5 min at 72°C.

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48 Amplification reactions were carried out in volumes of 25 µL containing 3 µL (10 pg-  
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50 10 ng) of template DNA, 1.25 µL of each primer (20 µM), 2.5 µL of 10x PCR buffer, 1  
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52 µL of MgCl<sub>2</sub> (50 mM), 0.25 µL of dNTPs (100 mM) and 0.2 µL of Taq DNA  
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54 polymerase (5 U/µL) supplied by the manufacturer (Biotools Madrid, Spain). PCR  
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56 products were detected in 2% agarose ethidium bromide gels in TAE 1X buffer (Tris-  
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3 acetate 40 mM and EDTA 1.0 mM). The 100 bp DNA ladder (MBI Fermentas, Vilnius,  
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5 Lithuania) was used as molecular size marker.  
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#### 10 *Detection of fungal spores in wheat flour*

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12 Molds are found in dry stored food and grains mostly as dormant structures, e.g., spores.  
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14 To test the ability of the PCR to detect *A. flavus* in food, different lots of commercially  
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16 available ground wheat flour (1g each) were inoculated separately with  $10^2$  or  $10^6$   
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18 spores per g of *A. flavus* ITEM 4591. The flour was then resuspended in 5 ml of  
19  
20 Sabouraud (Scharlau Chemie, Barcelona, Spain) with 1ml/l chloramphenicol and  
21  
22 incubated in 10 ml sterile Falcon tubes on an orbital shaker at 28°C and 150 rpm. The  
23  
24 samples were incubated for 8, 16, 24 and 48 h respectively. Non-inoculated flour  
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26 samples were used as negative control. After incubation, the tubes were subsequently  
27  
28 centrifuged (5 min at 5000 rpm), the supernatant discarded and the pellets were frozen  
29  
30 in liquid nitrogen. Total DNA was extracted and PCR reactions were carried out using  
31  
32 the same protocols described above for pure cultures. All the experiments were made in  
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34 triplicate.  
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## 43 **Results**

### 44 *PCR specificity with FLA1/FLA2*

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46 Partial sequences of the ITS1-5.8S-ITS2 regions of *A. flavus* and other related  
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48 *Aspergillus* species obtained in this and previous works and those retrieved from Data  
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50 Bases (more than 25) were aligned. The design of a PCR assay is limited by the  
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52 conserved regions available in the alignments and the suitable size of the amplicon to be  
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54 easily detected in conventional agarose gels using ladder markers. A pair of primers,  
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56 FLA1 and FLA2, specific to *A. flavus* was designed on the basis of the sequence  
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3 alignment. All the *Aspergillus* strains listed in Table I were tested for amplification  
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5 using the primer pair FLA1 and FLA2. A single fragment of about 500 bp was only  
6  
7 amplified when genomic DNA from *A. flavus* strains was used (Figure 1), but not from  
8  
9 other *Aspergilli*, including *Aspergillus* section *Flavi* species (*A. tamarii*, *A. nomius*, *A.*  
10  
11 *bombycis* and *A. parasiticus*) or other *Aspergillus* (such as *A. ochraceus* or *Aspergillus*  
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13 section *Nigri*). No product was observed with genomic DNA from other genera. Non-  
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15 specific products were not observed in any case. Control amplifications of the genomic  
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17 DNA with primers ITS1 and ITS4 were positive for all the strains analysed.  
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#### 25 *PCR studies with inoculated wheat flour*

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27 In order to test the functionality of the method under practical conditions, an enrichment  
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29 procedure was developed. In this procedure, Sabouraud-Chloramphenicol broth was  
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31 added to inoculate wheat flour which was then incubated. The primer pair generated for  
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33 the amplification of *A. flavus* was tested on samples after 8, 16, 24 and 48 h of  
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35 incubation. Two levels of inoculated spores,  $10^2$  and  $10^6$  spores per g, were tested.  
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37 Specific amplification was observed only for DNA extracted from wheat inoculated  
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39 with *A. flavus* ITEM 4591 (Figure 2). A clear amplification product was observed when  
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41 wheat flour was inoculated with  $10^2$  or  $10^6$  spores per g after 16, 24 and 48 h of  
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43 incubation. No amplification products were obtained when DNA was extracted from  
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45 non-incubated samples or incubated for 8h. The triplicates of the experiments showed  
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47 no significant variation in the results.  
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#### 55 **Discussion**

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57 In this work, we have developed a PCR protocol specific for the detection of *A. flavus*  
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59 to be used in a food matrix frequently contaminated with aflatoxins and other related  
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3 *Aspergillus* species. This is the first protocol to our knowledge to specifically detect *A.*  
4 *flavus* by PCR amplification using a multycopy region (ITS). Discrimination of *A.*  
5 *flavus* from *A. parasiticus* is important because they produce different secondary  
6 metabolite profiles. The aflatoxins, cyclopiazonic acid, versicolorin and  
7 sterigmatocystin can all be produced by *A. flavus* while *A. parasiticus* is not known to  
8 produce cyclopiazonic acid and sterigmatocystin (Wilson et al. 2002). Moreover, *A.*  
9 *flavus* was the prevalent species among *Aspergillus* section *Flavi* in most of the studies  
10 performed in several commodities (Horn and Dorner 1999; Trucksessy et al. 2002;  
11 Vaamonde et al. 2003; Bankole et al. 2004; Melki Ben Fredj et al. 2006).

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27 Although several PCR-based methods have been developed to detect aflatoxigenic fungi  
28 using aflatoxin biosynthetic genes they do not discriminate between *A. parasiticus* and  
29 *A. flavus*, and the amplification signal obtained is strong for *A. parasiticus* but it is weak  
30 for *A. flavus* (Geisen 1996; Shapira et al. 1996; Zachová et al. 2003). This could be due  
31 to the similarity of the aflatoxin biosynthesis genes used as targets for specific primers  
32 in these two species (Yu et al. 1995; Scherm et al. 2005). The existing variability in ITS  
33 region we have used seemed to be sufficient to discriminate among these closely related  
34 species and, on the other hand, provided higher sensitivity than single copy genes.  
35 Indeed, the PCR assay developed for *A. flavus* did not cross react with *A. parasiticus*.

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51 Moreover, robustness of the specific PCR assay for *A. flavus* was high according to the  
52 positive PCR amplification obtained with a wide range of template concentration (10pg-  
53 10ng) using either DNA from pure cultures or from contaminated sample. It has been  
54 estimated a sensitivity for multycopy sequences above 5.5 pg and for single copy  
55 sequences above 87.5 pg on agarose gel (Jurado et al. 2006). A similar approach was  
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3 followed by Sugita et al. 2004 to identify *A. flavus* and another pathogenic species of  
4 the genus *Aspergillus*. They used ribosomal DNA and its flanking regions (including  
5 ITS1 region), but the efficiency of amplification in the case of *A. flavus* was still low.  
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10 The FLA1/FLA2 primers seemed to be more efficient to specifically amplify the *A.*  
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Other approaches, such as PCR-RFLPs of ITS region, have been followed to discriminate both species, but are more laborious (Somashekar et al. 2004).

However, this protocol does not allow the discrimination between *A. flavus* and *A. oryzae*. Molecular methods, including isoenzyme analyses and DNA/DNA hybridization studies, and DNA sequencing and comparison of the whole genome have confirmed that both species are almost genetically identical (Kurtzman et al. 1986, Egel et al. 1994, Geiser et al. 2000, Payne et al. 2006, Hedayati et al. 2007), with the exception of few differences in genes involved aflatoxin biosynthetic pathway (Geiser et al. 2000). On the other hand, while *A. flavus* is a common environmental organism, *A. oryzae* is a 'domesticated' fungus and it would not be present in the niches where *A. flavus* occurs.

Specific diagnosis in pure cultures is generally easier than directly on commodities where a complex mycobiota is present, often containing closely related species and several compounds which may affect the efficiency and sensitivity of the detection (Rossen et al. 1992; Färber et al. 1997) and in some cases additional incubation of the food samples for several days is required (Chen et al. 2002). Since molds are found mostly as asexual spores or dried mycelia on dry food, which contain only small amounts of DNA and are resistant to cellular disruption for DNA extraction (Shapira et

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3 al. 1996), we have improved the sensitivity and applicability of our PCR protocol with  
4 an enrichment method to be used in wheat flour contaminated by spores. This integrated  
5 protocol allowed us to detect  $10^2$  spores per g after 16 h of incubation. This incubation  
6 time was shorter than the reported by other authors (Shapira et al. 1996), and the  
7 detection limit high in our study. Although genomic DNA of wheat and contaminating  
8 fungi may become severely damaged during the manufacturing process, PCR assays  
9 could be particularly suitable to detect fungal contamination in flour because of the  
10 small size of the target genomic DNA. Previous reports describing PCR assays with  
11 amplicons of similar length indicate successful amplification using DNA isolated from  
12 flour (maize) (Jurado et al., 2006; Somashekar et al., 2004). These evidences, and the  
13 high copy number of the target sequence, increase the probability of available template  
14 even after processing of wheat grains. On the other hand, very often contamination by  
15 post-harvest fungi occurs after processing of the seeds in the warehouse.  
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36 In conclusion, the PCR based protocol reported in this work is a rapid and powerful tool  
37 to detect *A. flavus* in certain food systems. The PCR assay and the sample preparation  
38 protocol were optimised to avoid the occurrence of false negative reactions.  
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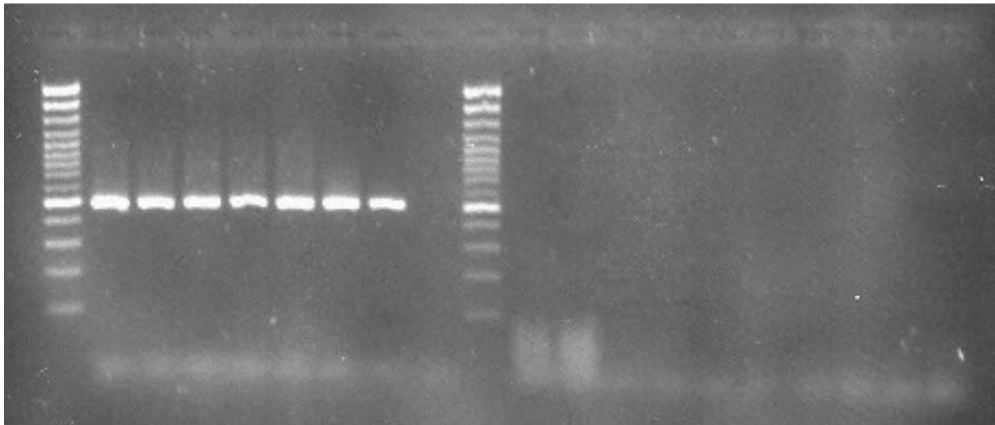
**Figure caption**

Figure 1. PCR amplification using primers FLA1/FLA2 and DNA from *Aspergillus flavus* strains. Lanes 1-7: CECT 2681, ITEM 4591, ITEM 4592, ITEM 5135, M2hip10, M1hip6, M12hip10, lane 8: Non-template control, lane 9: *A. parasiticus* Cab5dch6, lane 10: *A. tamarii* IHEM 16077, lane 11: *A. bombycis* IHEM 19289, lane 12: *A. ochraceus* CECT 2092, lane 13: *A. nomius* IHEM 18041, lane 14: *A. carbonarius* (CECT 2086), lane 15: *A. niger* (CECT 2091), lane 16: *A. tubingensis* (TR 20 1), lane 17: *Penicillium verrucosum* (CECT 2906), lane 18: *Cladosporium* sp. (CL.1). M: DNA molecular size marker.

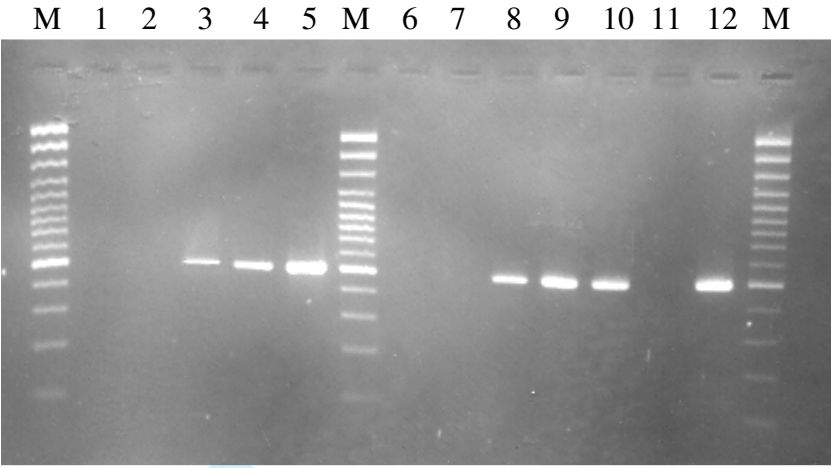
Figure 2. PCR-based detection of *A. flavus* using FLA1/FLA2 primers in wheat flour samples after enrichment. Lanes 1-5: Amplification product obtained using DNA after 0, 8, 16, 24 and 48 h of incubation, respectively, and inoculated with  $10^2$  spores of *A. flavus* per g. Lanes 6-10: Amplification product obtained using DNA extracted after 0, 8, 16, 24 and 48 h of incubation, respectively, and inoculated with  $10^6$  spores of *A. flavus* per g. Lane 11: Non-template control. Lane 12: Amplification product obtained using as template *A. flavus* ITEM 4591 DNA. M: DNA molecular size marker.

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Table I. Fungal strains analysed indicating, origin, species, host and the occurrence of PCR amplification product with primers FLA1 and FLA2.

Strains supplied by Dr. Sanchis (University of Lleida, Spain)(\*). Strains supplied by Dr. Moretti (CNR, Bari, Italy)(\*\*)

STRAIN	ORIGIN	SPECIES	HOST	FLA1-FLA2
M1hip6	Madrid (Sp)	<i>A. flavus</i>	Barley	+
M12hip10		<i>A. flavus</i>	Barley	+
M2hip10	Sevilla (Sp)	<i>A. flavus</i>	Barley	+
CECT 2681		<i>A. flavus</i>		+
ITEM 5135	Italy	<i>A. flavus</i>	Flour	+
ITEM 4592	France	<i>A. flavus</i>	Wheat	+
ITEM 4591	France	<i>A. flavus</i>	Wheat	+
ATCC 20043		<i>A. flavus</i>	Soil	+
NRRL 453 <sup>1</sup>		<i>A. flavus</i>	Brazil nut	+
NRRL 1957		<i>A. flavus</i>	Cellophane diaphragm	+
JMC 12721		<i>A. flavus</i>	Cottonseed	+
IHEM 16077	Belgium	<i>A. tamarii</i>	Bread	-
M6hip1	Albacete (Sp)	<i>A. tamarii</i>	Barley	-
IHEM 19289	Japon	<i>A. bombycis</i>	Silkworm disease	-
IHEM 18041	French Guiana	<i>A. nomius</i>	Mango tree	-
Cab5dch6	Albacete (Sp)	<i>A. parasiticus</i>	Barley	-
NRRL 502 <sup>1</sup>		<i>A. parasiticus</i>		-
NRRL 465		<i>A. parasiticus</i>		-
ATCC 56775		<i>A. parasiticus</i>		-
ATCC 22789		<i>A. parasiticus</i>		-
ATCC 15517 <sup>H</sup>		<i>A. parasiticus</i>		-
ATCC 22789		<i>A. parasiticus</i>		-
C31	Valladolid (Sp)	<i>A. ustus</i>	Barley	-
C32	Valladolid (Sp)	<i>A. ustus</i>	Barley	-
T.T.A.13	Zamora (Sp)	<i>A. fumigatus</i>	Grapes	-
R.T.A.16	Valladolid (Sp)	<i>A. fumigatus</i>	Grapes	-
CECT 2808		<i>A. terreus</i>		-
CECT 2907		<i>A. awamori</i>		-
CECT 2903		<i>A. versicolor</i>		-
CECT 2546		<i>A. sclerotiorum</i>		-
CECT 2091	Canada	<i>A. niger</i>		-
CECT 2574		<i>A. niger</i>		-
TR 20 1	Valladolid (Sp)	<i>A. tubingensis</i>	Wheat	-
TB 2 1	Soria (Sp)	<i>A. tubingensis</i>	Wheat	-
TB 5 2	Valladolid (Sp)	<i>A. tubingensis</i>	Wheat	-
350*	Spain	<i>A. carbonarius</i>	Grapes	-
190*	Spain	<i>A. carbonarius</i>	Grapes	-
CECT 2086		<i>A. carbonarius</i>		-
ITEM 4158**	Italy	<i>A. japonicus</i>	Grapes	-
ITEM 4685**	Portugal	<i>A. japonicus</i>	Grapes	-
CECT 2092		<i>A. ochraceus</i>		-
CECT 2948		<i>A. ochraceus</i>		-
CECT 2093		<i>A. ochraceus</i>		-
CL.1	Valladolid (Sp)	<i>Cladosporium sp</i>	Grapes	-
CECT 2906		<i>Penicillium verrucosum</i>		-