Quality Assurance and Safety of Crops & Foods, 2015; 7 (3): 375-383

Wageningen Academic
Publishers

A simple PCR for detection of *Aspergillus flavus* in infected food

H.L.T. Trinh, D.T. Anh, P.M. Thong and N.T. Hue*

International University – Vietnam National University in HCMC, School of Biotechnology, block 6, Linh Trung Ward, Thu Duc District, 70000 Ho Chi Minh, Vietnam; nthue@hcmiu.edu.vn

Received: 12 July 2013 / Accepted: 28 February 2014

RESEARCH ARTICLE

Abstract

The protocol for detecting *Aspergillus flavus* was based on a simple polymerase chain reaction with high sensitivity and specificity. The specific primers were designed relying on the aflatoxin biosynthesis gene clusters. The limit of detection (LOD) is 2 ng/assay for single DNA and 3.75 ng/assay for mixed DNA. The direct extraction of DNA from the infected samples is used for the protocol. The LOD of the protocol in the detection of *A. flavus* is 40 ng of total DNA. The results also indicated that the sensitivity and specificity of the optimised protocol was twenty times higher than that of the conventional morphological method. This protocol could be used for monitoring *A. flavus* in stored food or feed, especially in the detection of a potential aflatoxigenic risk in stored peanut and corn kernels, using a rapid, effective and high-throughput method, even in the case of low-level infections.

Keywords: aflatoxin, aflatoxin biosynthesis gene clusters, limit of detection

1. Introduction

Mycotoxins are naturally-occurring substances produced as secondary metabolites by microbial fungi (moulds). They are capable of impairing crops and other food commodities in both the pre- and post-harvest stage (Abdel-Hadi *et al.*, 2010; Abdin *et al.*, 2010). Mycotoxin contamination is a global problem in food security due to its toxicity. More than 300 mycotoxins have been discovered to date, however, the most toxic group is that of aflatoxins.

Aflatoxins are immunosuppressive, carcinogenic and mutagenic secondary metabolites. They are classified into 4 main types, B_1, B_2, G_1 and G_2 in which B_1 is the main agent causing disease in human and animals that have unfortunately consumed fungus-contaminated food or feed (Bhatnagar *et al.*, 2006; Dehghan *et al.*, 2008; Kabak, 2010; Pattron, 2006; Putri *et al.*, 2003; Tran-Dinh *et al.*, 2009). For a long time, they have been known as the cause of 4 distinct effects including acute liver damage, liver cirrhosis, induction of tumours and teratogenic effects. It has been demonstrated that aflatoxin B_1 was the most potent liver carcinogen known in a variety of animal species. Aflatoxin B_1 has been linked to human hepatocellular carcinoma in several regions of Africa and Southeast Asia (Wang and Groopman, 1999). M_1 and M_2, compounds with lower toxicity, are found to be excreted in milk, when B_1 and B_2 are ingested by lactating cows. Children who consume large quantities of milk are susceptible to long-term exposure to aflatoxins. In developing countries, where detection and decontamination policies are impractical, aflatoxin contamination is predominantly a food safety issue. Because of the deleterious health effects of aflatoxins, their doses have been strictly regulated. By the end of 2003, approximately 100 countries had specific guidelines for mycotoxins; 54.5% of the world's inhabitants live in 26 countries in Asia/Oceania under regulations monitoring all aflatoxin contamination in food and contamination of B_1 in feed (Van Egmond and Jonker, 2004; Van Egmond *et al.*, 2007).

Aflatoxins are mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*, and cause aspergillosis in animals and humans (Putri *et al.*, 2003). The quest for knowledge about potential aflatoxin problems made *A. flavus* the most widely reported foodborne fungus despite its important role in nature (Pitt and Hocking, 2009). In many reports, this mould was classified as a plant pathogen; particularly in developing seeds such as corn, peanuts, cottonseed, and tree nuts (Bhatnagar *et al.*, 2006; Kabak, 2010).

ISSN 1757-837X online, DOI 10.3920/QAS2013.0308

A. flavus grows well in regions with tropical, subtropical or warm temperate climates (latitudes 16-35°) and perennially infects important crops (Passone *et al.*, 2010). It will usually be able to produce aflatoxin at 12-40 °C (Schwartzburg, 2005). In addition to the temperature requirement (10-42 °C), the fungus also requires a pH ranging from 2 to 10.5. It was noticed that the optimal water activity in food or feed required for its growth and aflatoxin production are approximately 0.99 and 0.85, respectively (Abdel-Hadi *et al.*, 2010; Scheidegger and Payne, 2005).

The presence of aflatoxins in food causes problems in trading, leading to economic loss, due to the difference in accepted limits for aflatoxins by local authorities (Aquino *et al.*, 2008; Shiferaw *et al.*, 2011; Stroka and Anklam, 2002). For example, the US Food and Drug Administration only allows up to 20 ng of aflatoxin B_1, B_2, G_1 or G_2 per gram weight of human food or animal feed (Surendranatha Reddy *et al.*, 2011). With such a policy, 21% of groundnut and maize in India would become unhealthy for human consumption and would be rejected by the USA since they contain 30 μg aflatoxins per kilogram of food. As a result, over a decade, the export of groundnut products from India has declined by 285 metric tons (equivalent to the loss 10 of million USD) (Chen *et al.*, 2005). The aflatoxin-contaminated food has, therefore, raised serious concerns at both the national and international level.

It is difficult to distinguish *A. flavus* from other *Aspergillus* using conventional methods, which are mainly based on morphological isolation or immunological features. These methods are time-consuming and labour-intensive; they also require facilities and mycological expertise (Pitt and Hocking, 2009). Other recently developed methods, including immunosensors, thin layer chromatography, high performance liquid chromatography and real-time polymerase chain reaction (PCR), were able to overcome those drawbacks but are either too complicated or expensive (Abbas *et al.*, 2004; Sun *et al.*, 2012; Turner *et al.*, 2009). The use of PCR assay has therefore been nominated as an ideal option for detecting *A. flavus* to minimise or exclude those limitations. Several PCR methods using universal hybridisation, oligonucleotide probes or microarray have been used for the pathogen detection but they are still complicated or not routinely used (Abdin *et al.*, 2010). A simple method needs to be developed that has high specificity and sensitivity, is less labour-intensive and requires less expertise.

In this study, a PCR assay with high specificity and sensitivity was developed which can overcome the limitations of recent methods and can be used for the detection of *A. flavus* in food to control the spread of aflatoxin-infected food or the accumulation of aflatoxins during a lifetime.

2. Materials and methods

Fungi strains

12 isolates of filamentous fungi, representing six *Aspergillus* and *Aspergillus*-related species were used in this study. All isolates of *A. flavus* (VTCC-F-160, VTCC-F-898), *Aspergillus oryzae* (VTC-F-910, VTCC-F-912), and *A. parasiticus* (VTCC-F-1130, VTCC-F-1132, ATCC-16404) were obtained from the Institute of Microbiology and Biotechnology, Vietnam National University, Hanoi, Vietnam. *A. flavus* (AF.IV26.1) and *Aspergillus niger* (ATCC-16404) were obtained from the Pasteur Institute, HCM city, and American type culture collection, respectively; *A. flavus* (TN1), *Aspergillus candidus*, and *Penicillium aethiopicum* (TNTT) were obtained from Quality assurance and testing centre 3 (Quatest3), Ho Chi Minh City, Vietnam. Fungal strains were maintained on potato dextrose agar (Dehghan *et al.*, 2008; Pattron, 2006). They were incubated in Blakeslee's malt extract broth (2% malt extract, 2% dextrose, 0.1% peptone) at 25 °C, 120 rpm for 3 days before DNA were extracted.

Infected samples

The dried peanut and corn kernels were inoculated with VTCC-F-898 spores for 7 days and identified the day before seeing fungal grow on the surface of samples.

Sample treatments

The infected samples were processed using two different treatment methods. With the direct method, three, five, and seven kernels from each of the samples were picked up randomly, and placed in 10 ml of 0.85% sodium chloride solution, centrifuged at 1,500 rpm, for 5 min at room temperature to remove *A. flavus* from the infected kernels. These *A. flavus* containing solutions were used for DNA extraction. A similar procedure was performed for the indirect method. However, before isolating the fungus, the kernels were enriched in 10 ml of malt extract broth for 24 h at room temperature. To generate reliable data, we performed 3 to 7 replicates for each sub-sample.

DNA extraction

The collected solutions were centrifuged at 10,000 rpm for 10 min, followed by 13,000 rpm for 5 min, both at 25 °C. Sterilised glass powder was added to the tubes at a ratio of 1:3 (gram of microorganism pellet:gram of glass powder), crushed up together by sterilised plastic sticks for 3 min. After that, 1 ml SDS lysis buffer (10 mM Tris-HCl, 20 mM EDTA, 5% SDS, 0.7 M NaCl) was added to each tube, swirled for 2 min before adding 10 μl proteinase K 2 mg/ml (Promega, Madison, WI, USA) and 5 μl 2-mercapto-ethanol followed by heat shock at 65 °C for 30 min, -80 °C for 5 min and 65 °C for 10 min. After centrifugation (5 min, 8,000 rpm, 25 °C), the supernatant was transferred to a new sterile tube. An equal volume of chloroform was added. The tube was inverted several times then centrifuged for 5 min, 13,000 rpm, 25 °C. The aqueous layer was transferred to a new tube and extracted again with an equal volume of chloroform. After centrifugation, the supernatant was transferred to another new tube, mixed with 0.6 volumes of cold isopropanol and 0.08 volumes of 7.5 M ammonium acetate, continuously incubated at -20 °C for 30 min, and then centrifuged at 4 °C, 13,000 rpm for 15 min. The supernatant was discarded and the isolated pellet was washed in 100 µl ice-cold ethanol 70%. The tube was spun again at 13,000 rpm, 4 °C, and 5 min. After removing the supernatant, the DNA pellet was air-dried overnight, then dissolved in 100 µl TE buffer.

DNA quantity and quality were measured by NanoDrop™ 1000 (Labwrench, Ontario, Canada). The high purity was confirmed if the ratio of the absorbance at 260 and 280 nm (A260/280) was in the range of 1.7-1.9.

Primers design

Aflatoxin biosynthesis gene cluster of *A. flavus* (isolate AF13, AF36, AF70), *A. oryzae* (isolate RIB40), *A. parasiticus*, and *A. niger* were obtained from Genbank (National Center for Biotechnology Information (NCBI), US National Library of Medicine, Bethesda, MD, USA; www.ncbi.nlm.nih.gov/nuccore) and aligned with BioEdit software (version 7.0.5.3; Ibis Biosciences, Carlsbad, CA, USA). Primers were designed based on nucleotide sequences that are unique to *A. flavus*. Primers to be designed were compared against all other oligonucleotides available online with the basic local alignment search tool algorithm (BLAST, NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Optimizing PCR

The annealing temperature for gradient PCR amplification ranged from 54 to 69 °C (54, 57, 60, 63, 66 and 69 °C). Each PCR reaction consisted of 1.5-2 µl of the isolated DNA and 23 µl PCR master mix containing 12.5 µl Top Taq DNA polymerase (Qiagen, Venlo, the Netherlands), 5 mM primers and adjust with distilled water. The PCR thermal cycler setup was: 5 min at 94 °C followed by 35 amplification cycles at 94 °C for 30 s, annealing temperature for 30 s, 72 °C for 30 s, and then 72 °C for 3 min for the final extension. The PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide at 80 V for 20 min (MP 3AP; Satori Bio, Berkeley, CA, USA). The result was observed under ultraviolet (UV) light (GelDoc-It 310; UVP, Upland, CA, USA) and compared with the marker (EZ Load™, 100 bp; Bio-rad, Hercules, CA, USA).

3. Results

The protocol for detection of *A. flavus* is developed in two stages. The first involves designing and examining the assay with the pure *A. flavus* and other *Aspergillus* species. The second consists of evaluating the designed PCR assay by applying it to detect *A. flavus* in the infected food which includes optimizing the sample treatment method to provide samples for the designed PCR assay.

Validate primers and PCR condition

BLAST result for forward primer (AFLA-F-5'-GGTGGTGAAGAAGTCTATCTAAGG-3') showed that all 24 nucleotides of AFLA-F match oligonucleotide of *A. flavus* (identities = 24/24) and no gap was found (gaps = 0/24). No other 100% matches and no gap was found on the other species. On the contrary, BLAST result for reverse primer (AFLA-R-5'-AAGGCATAAAGGGTGTGGAG-3') showed that all 20 nucleotides of AFLA-R match oligonucleotide of both *A. flavus* and *A. oryzae* (identities = 20/20) with no gap was found (gaps = 0/20). This indicated that the designed forward primer is specific for *A. flavus* only. The PCR product is a 413 bp.

PCR assay

Figure 1 shows the results of PCR product checking from the gradient PCR assay on agarose gel; the presence of the band at 413 bp indicated the expected PCR product. The annealing temperatures of 54, 57, 60 and 63 °C revealed sharply amplified products; a greater intensity was especially apparent at 60 °C. However, when the temperature increased to 66 °C, a faint PCR product of 413 bp was observed. No amplification product was observed

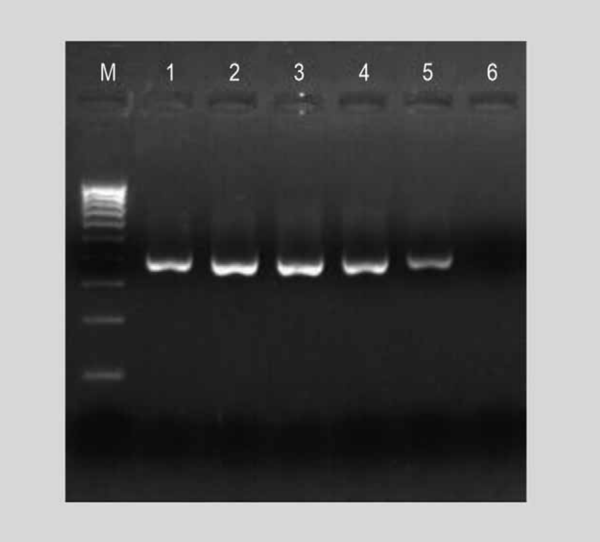

Figure 1. Checking annealing temperature of primers. Lane 1 to 6: 54, 57, 60, 63, 66 and 69 °C.

at 69 °C (Figure 1). Given that the most specific annealing temperature falls between 63 to 66 °C, 65 °C was selected for all further amplifications.

Specificity analysis

A total of 12 *Aspergillus* isolates (*A. flavus, A. oryzae, A. parasiticus, A. niger* and *A. candidus*) and an *Aspergillus* related isolate (*Penicillium aethiopicum*) were used for testing specificity of primers. The samples were prepared independently with an equal amount of DNA (70 ng/µl) from individual strains. DNA mixture including all 12 strains was also tested.

The result for testing the specificity of the designed primers is shown in Figure 2. The result indicated that only DNA from *A. flavus* could be amplified (lane 2 to 5), no matter what the presence of other species (lane 1). No PCR product from other fungal species (non-*flavus Aspergillus* and *Penicillium*) was obtained (lane 6 to 13). Only *A. flavus* species was detected by PCR using the designed primer set. Thus the primer set is specific for detection of *A. flavus.*

Sensitivity analysis

The limit of detection (LOD) was examined based on the ability of the assay to detect *A. flavus* in the VTCC-F-898 DNA solution with a series dilution of DNA concentration 15, 7.5, 3.75, 2, 1, 0.5, and 0.25 ng. The LOD confirmation was performed based on the DNA solution of a mixture of 12 known *Aspergillus* spp. with series dilution of total DNA concentration 30, 15, 7.5, 3.75, 2, 1 and 0.5 ng

For VTCC-F-898 DNA solution (Figure 3), the PCR reaction with 15 ng DNA template gives the best result where the band appears sharpest (lane 1). As the concentration decreases, the bands become fainter until insignificant at 1 ng. With too low a concentration of DNA template, the PCR assay cannot detect the presence of *A. flavus* in the solution. Thus, the LOD of this PCR assay is 2 ng/assay.

For the DNA solution mixture, the band appeared sharpest at 15 ng total DNA and became fainter at lower concentrations (Figure 4). No PCR product was obtained at 2 ng DNA mixture. The LOD of PCR assay in detection of *A. flavus* using DNA mixture was concluded at 3.75 ng/assay.

Preparing DNA samples from infected foods

When processing the infected peanuts with the direct method, the average amounts of DNA extracted from three, five, and seven kernels from 5 replicates were 10.12±0.45, 35.72±3.73 and 15.55±2.76 ng/µl, respectively (Figure 5). For comparison, non-infected peanuts were also included and used as controls. DNA concentrations from these controls were 5.42±0.007 ng/µl for 3 kernels, 7.1±4.15 ng/µl for 5

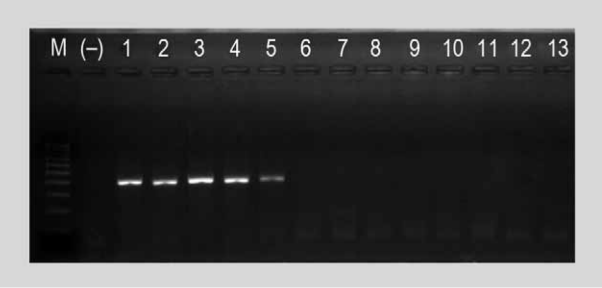

Figure 2. The specificity of primers used for detection of *Aspergillus flavus.* M = marker; (–) = negative control; lane 1 = DNA mixture; lane 2-13 = *A. flavus* VTCC-F-160, *A. flavus* VTCC-F-898, *A. flavus* AF.IV26.1, *A. flavus* TN1, *Aspergillus oryzae* VTCC-F-910, *A. oryzae* VTCC-F-912, *Aspergillus parasiticus* VTCC-F-1130, *A. parasiticus* VTCC-F-1132, *A. parasiticus* VTCC-F-1159, *Aspergillus niger* ATCC-16404, *Aspergillus candidus*, *Penicillium aethiopicum* TNTT.

Figure 3. Sensitivity analysis of PCR assay in detection of *Aspergillus flavus* based on VTCC-F-898 DNA solution. M = marker; (–) = negative control; lane 1-7 = 15, 7.5, 3.75, 2, 1, 0.5 and 0.25 ng of VTCC-F-898 DNA.

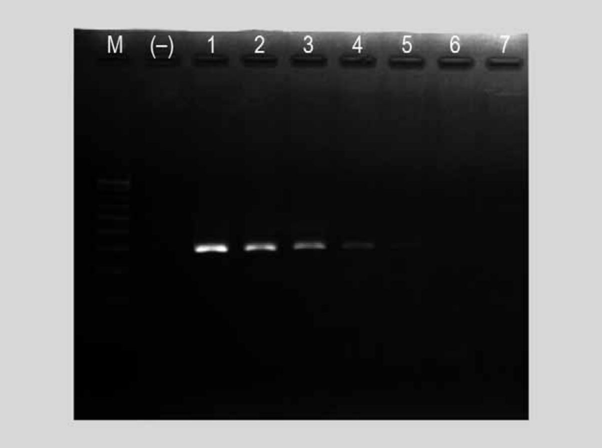

Figure 4. Sensitivity analysis of PCR assay in detection of *Aspergillus flavus* using the DNA solution mixture. M = marker; (–) = negative control; lane 1-7 = 30, 15, 7.5, 3.75, 2, 1, and 0.5 ng of total DNA.

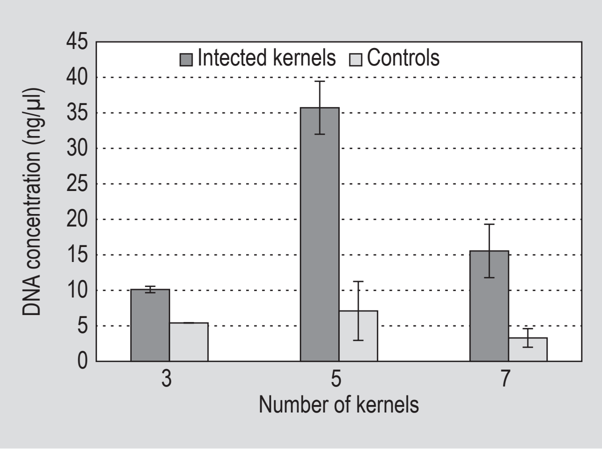

Figure 5. DNA extracted directly from 3, 5 and 7 peanuts kernels.

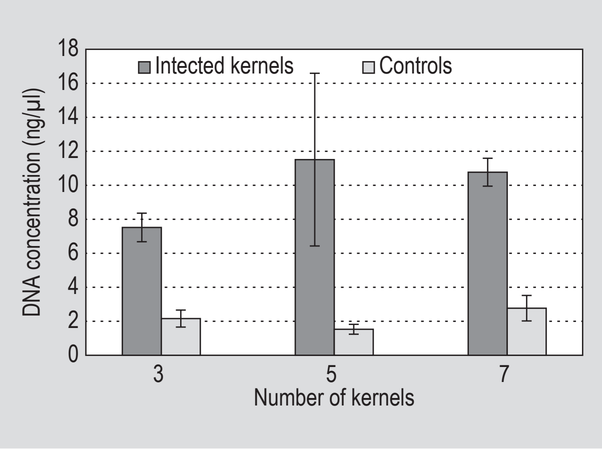

Figure 6. DNA extracted directly from 3, 5 and 7 corn kernels.

kernels, and 3.3±1.31 ng/µl for 7 kernels (Figure 5). For corn kernels which had been inoculated with *A. flavus* for 3 days, the DNA concentrations isolated from 3, 5 and 7 kernels from 4 replicates in succession were 7.52±0.84, 11.51±5.08 and 10.77±0.82 ng/µl (Figure 6). Meanwhile, DNA harvested from the corresponding non-inoculated corns was 2.16±0.5, 1.53±0.29 and 2.77±0.75 ng/µl, respectively (Figure 6). There were significant differences between average amounts of DNA in groups of samples using two-tailed t-test for comparison (Table 1).

Once the enrichment step was included, the extracted amounts of DNA were 10-27 times higher for the infected kernels and 17-64 times higher for the control samples. With infected peanuts, the quantified DNA values of these sub-samples from 5 replicates were 384.51±37.95 (3 kernels), 565.68±27.18 (5 kernels), and 955±157.76 ng/µl (7 kernels) whereas those of their control partners were 122.3±31.37 ng/µl, 158.58±25.24 ng/µl and 267.72±28.38 ng/µl (Figure 7). A similar result was seen for the corn kernels whereby total DNA concentrations from 5 replicates were 893.16±110.18 ng/µl (3 infected kernels), 711.87±51.62 ng/µl (5 infected kernels) and 801.88±56.78 ng/µl (7 infected kernels). For control samples, DNA yields were 450.57±135.27 ng/µl (3 kernels), 212.35±42.65 ng/µl (5 kernels) and 342.35±44.26 ng/µl (7 kernels) (Figure 8).

In both kinds of substrates, peanut and corn, the average amount of DNA from enriched infected samples was significantly different from the average amount of DNA from control samples. All results had $P<0.05$ (Table 2). This also suggested that the samples could be successfully infected.

Detection of *Aspergillus flavus* by designed PCR assay

2 µl isolated DNA from sub-samples of both infected samples and controls by direct method and 2 µl of DNA after diluting 10 times obtained by indirect method were used as DNA templates for PCR reactions. According to Figure 9, with the indirect method (lanes 1-6), PCR products loaded into all six lanes showed the bands; the DNA concentration of *A. flavus* in these lanes increased in conjunction with a higher number of infected kernels that were used for extracting the fungal DNA. The PCR products in the third and sixth lane, amplified from DNA

Table 1. Statistical analysis of extracted DNA amount from groups of samples in direct treatment.

		Average sDNA[1]	Average cDNA[2]	*P*-value[3]
Peanut	3 kernels	10.12±0.45	5.42±0.007	*P*=0.0005
	5 kernels	35.72±3.73	7.1±4.15	*P*=0.005
	7 kernels	15.55±2.76	3.3±1.31	*P*=0.045
Corn	3 kernels	7.52±0.84	2.16±0.5	*P*=0.01
	5 kernels	11.51±5.08	1.53±0.29	*P*=0.004
	7 kernels	10.77±0.82	2.77±0.75	*P*=0.009

[1] Average sDNA = average DNA amount extracted from infected samples (mean ± standard deviation) (ng/µl).
[2] Average cDNA = average DNA amount extracted from control samples (mean ± standard deviation) (ng/µl).
[3] *P*-value: using two-tailed t-test, *P*=0.05.

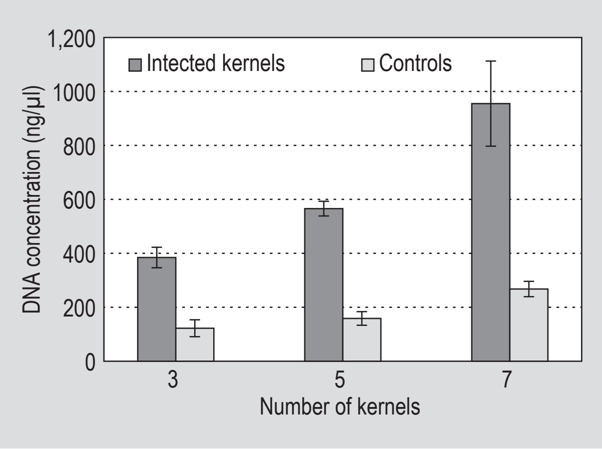

Figure 7. DNA obtained from peanut samples using indirect method.

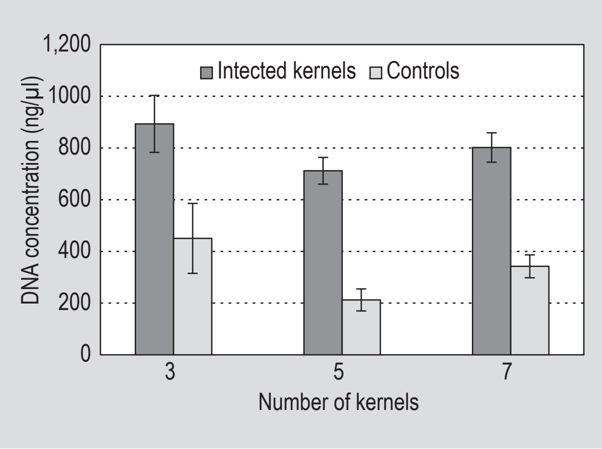

Figure 8. DNA obtained from corn samples using indirect method.

extracted from 7 infected peanut kernels and 7 infected corn kernels, respectively, had the bands with highest intensities. It was also noted that no band was seen in the control lane, demonstrating that there was no DNA template for amplification by PCR (Figure 9).

With direct method (lanes 7-12), only one dim band of PCR product at lane 9 appeared while no bands were seen in the remaining lanes although we intentionally chose samples with similar DNA concentrations for the PCR performance (i.e. to provide 21-40 ng DNA template) (Figure 9). Notably, lane 9 contained the PCR product amplified from the highest amount of DNA template (approximately 40 ng) among the samples.

This result supported the fact that the LOD by PCR assay in detecting *A. flavus* on peanut and corn kernels was from 40 ng of original DNA; and it also led to the second PCR reaction whereby the volume of DNA template for PCR should be increased twice in comparison to the first PCR if DNA was extracted by the direct method. Indeed, Figure 10 confirmed that, after using a double volume of isolated DNA for each PCR assay, PCR products were clearly seen on the gel for all sub-groups (3, 5 and 7 kernels). It has been noticed that although this study was not able to quantify accurately the proportion of target's DNA in the total DNA, the successful detection implied that the developed PCR should be relevant to identify *A. flavus* even when this fungus is present in the stored food or feed in low quantities.

The DNA purity obtained from either treatment was good since all the measurements were within the accepted range (A_{260}/A_{280} = 1.8-2.0). However, the direct method seemed to be more appropriate for checking the sensitivity and specificity of PCR assays at a later stage than the indirect method. Firstly, the former was less time-consuming. Secondly, the quantities of isolated DNA were less variable (smaller standard error values) but still sufficient for detection by the PCR assay. In contrast, when the enrichment step was included, DNA concentrations were significantly increased but less consistent, ranging from 385-960 ng/µl. This also implies that the more DNA is obtained, the more contamination might occur.

Table 2. Statistical analysis of extracted DNA amount from groups of samples in indirect treatment.

		Average sDNA[1]	Average cDNA[2]	*P*-value[3]
Peanut	3 kernels	384.51±37.95	122.3±31.37	*P*=0.01
	5 kernels	565.68±27.18	158.58±25.24	*P*<0.05
	7 kernels	955±157.76	267.72±28.38	*P*=0.006
Corn	3 kernels	893.16±110.18	450.57±135.27	*P*=0.07
	5 kernels	711.87±51.62	212.35±42.65	*P*=0.001
	7 kernels	801.88±56.78	342.35±44.26	*P*=0.02

[1] Average sDNA = average DNA amount extracted from infected samples (mean ± standard deviation) (ng/µl).
[2] Average cDNA = average DNA amount extracted from control samples (mean ± standard deviation) (ng/µl).
[3] *P*-value: using two-tailed t-test, *P*=0.05.

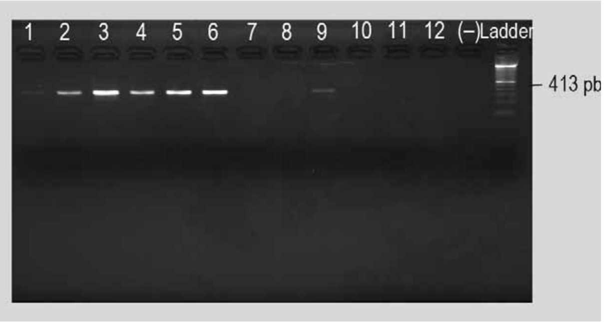

Figure 9. DNA validation: DNA extracted from direct (lanes 7-12) and indirect methods (lanes 1-6) by PCR assay. Lanes 1-3, 7-9 = DNA from 3, 5, 7 infected peanut; lanes 4-6, 10-12 = DNA from 3, 5, 7 infected corn kernels; lane (–) = negative control.

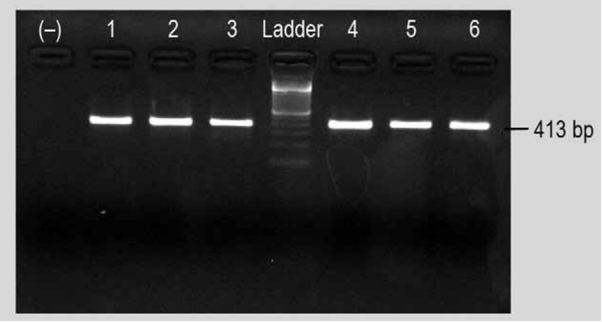

Figure 10. DNA confirmation: DNA extracted from direct method with double amount of template in PCR assay. Lanes 1-3 = extracts from 3, 5, and 7 infected peanut; lanes 4-6 = extracts from 3, 5, and 7 infected corn kernels; lane (–) = negative control.

4. Discussion

To develop specific primers for the identification of a unique target region, emphasis was placed on the aflatoxin biosynthesis gene cluster, which contains highly conserved sequences with some significantly variable regions. The alignment results on this cluster revealed a high degree of homology among the *Aspergillus* species. This accounts for the identities of all 20 nucleotides of AFLA-R on *A. flavus* and *A. oryzae.* The use of AFLA-F and AFLA-R as a set of primers for the PCR assay can discriminate *A. flavus* from the other *Aspergillus* species. This work aims to develop a highly specific test to allow the detection of *A. flavus* in the early stage of contamination. Additionally, because mycotoxin biosynthesis is a complex process that can be influenced greatly by environmental factors (Tran-Dinh *et al.*, 2009), it is unlikely that aflatoxin will be detected using this method. The test enables the prediction of aflatoxins via the presence of *A. flavus* even if *A. flavus* was killed in some cases. In this work, the PCR method has proved its advantages over morphological identification. The PCR method gave a better result in terms of accuracy, speed, and affordability. The PCR assay was developed to detect the presence of *A. flavus.* In solution with *A. flavus* only or with other *Aspergillus,* the PCR assay had been shown to have the necessary high sensitivity and specificity. With a very small amount of *A. flavus* DNA, the PCR assay can detect it easily. This also indicated that the designed primers, in the specific target, are very specific for detecting and distinguishing *A. flavus* from other fungi. The theory based on a specific target gene and the practical result had proved the success of the PCR assay in detecting *A. flavus* among others.

To use this designed PCR assay for the detection of *A. flavus* in real food where it may be contaminated with other organisms' DNA, a DNA extraction method was also optimised to get a good DNA solution as the template for the PCR assay. There are several methods for treating food samples to get target DNA for PCR. In this study, the direct and indirect methods were examined and the results showed that the quantities of the isolated DNA from both methods were variable depending on the change in the number of kernels used to harvest the fungus. However, apart from the results of DNA isolated from infected peanuts using the indirect method, the obtained DNA yield did not increase in proportion to the number of kernels that were used for extracting DNA. explaining possible explanation for this could be that even though *A. flavus* spore suspension and kernels were shaken regularly, amounts of *A. flavus* spores binding to each kernel's surface were still different whereas the kernels were randomly collected for the treatment experiment. As a result, fewer numbers of kernels highly contaminated with *A. flavus* were able to provide a larger amount of isolated DNA in comparison to using higher number of kernels that were less infected. In addition, a certain amount of DNA collected from the non-infected samples indicated that DNA from sources other than the target fungal DNA could also appear in the total DNA solution. The contaminated DNA could be from other types of microorganisms attaching to the kernels. It is likely that, since the kernels were not spread well enough in an Erlenmeyer flask, 30-min UV light exposure could not completely kill all microorganisms before they were used for the experiments. This was supported by an increase in DNA yield obtained from control samples if the enrichment step was included.

The infected kernels used for subgroups in this study, of which the weights were 0.5-1.5 g, provided a big enough source of *A. flavus* so that its DNA could be detected readily by the PCR assay. These quantities are considered approximately 20 times less than the required amount by the gold standard methods – morphological methods (Pitt and Hocking, 2009; Vietnam Ministry of Health, 2003). Therefore, this PCR assay was considered to be more sensitive in confirming the presence of *A. flavus* in food than the previous one. Moreover, both Figures 9 and 10 revealed that with lanes showing a visible band, only one sharp band at the size of 413 bp was seen. No smears or extra products were observed. Meanwhile, the control lane did not show any band even though double the volume of DNA had been used as template for the PCR. To ensure enough DNA was used for one PCR reaction since DNA concentrations of the control samples (both peanut and corn kernels) extracted by direct method were quite low, 10µl of isolated DNA was used for the assay. The obtained results indicated that all the control lanes had no bands on the agarose gel either (data not shown). This evidence again proved that the primer pair was designed specifically to identify *A. flavus*. Another advantage this PCR protocol offers is that it costs much less than diagnosis by other advanced methods such as immune-sensor, thin layer chromatography, high performance liquid chromatography and real-time PCR.

It is not influenced by environmental factors as it is used for detecting targets on stored food or feed. This protocol is relevant to track down the target from 0.5-1.5 g of infected food or at least 40 ng of the isolated DNA containing or total DNA. For this, it is much more sensitive and specific than the morphological methods that are being employed in our country. Consequently, this protocol would be expected to detect any stored commodities. Information derived from the tests would allow informed decisions about the storage life of peanut and corn kernels and the prevention strategies eventually needed, and would also reduce the economic losses for the agricultural countries, especially for those exporting their stored products to other countries.

5. Conclusions

In this study, new primers AFLA-F and AFLA-R were successfully designed relying on aflatoxin biosynthesis gene cluster and worked well at annealing temperature 65 °C. PCR assay used for identification of *A. flavus* performed effectively. The LOD were established with 2 ng/assay for single DNA and 3.75 ng/assay for mixture DNA.

As the direct method was successfully designed, the optimised protocol for detection of *A. flavus* on stored food or feed, especially dried peanut and corn, by application of PCR method with high sensitivity and high specificity, has been obtained. The protocol for preparing sample, extraction of DNA and then performing PCR assay could be used to detect *A. flavus* in infected food when the limitation of detection is 40 ng of total DNA.

Acknowledgements

The authors would like to thank QUATEST 3-HCMC (Quality Assurance and Testing Center 3-HCMC) for supporting the primary study. This study is funded by Vietnam National University Ho Chi Minh City (VNU) under grant number B2013-28-02.

References

Abbas, H.K., Shier, W.T., Horn, B.W. and Weaver, M.A., 2004. Cultural methods for aflatoxin detection. Journal of Toxicology 23: 295-315.

Abdel-Hadi, A., Carter, D. and Magan, N., 2010. Temporal monitoring of the nor-1 (aflD) gene of *Aspergillus flavus* in relation to aflatoxin B(1) production during storage of peanuts under different water activity levels. Journal of Applied Microbiology 109: 1914-1922.

Abdin, M.Z., Ahmad, M.M. and Javed, S., 2010. Advances in molecular detection of *Aspergillus*: an update. Archives of Microbiology 192: 409-425.

Aquino, S., Greiner, R., Konietzny, U., Hassegawa, R.H., Reis, T.A.D., Corrêa, B. and Villavicencio, A.L.C.H., 2008. Use of PCR for detecting *Aspergillus flavus* in maize treated by gamma radiation process. Food Biotechnology 22: 150-159.

Bhatnagar, D., Cary, J.W., Ehrlich, K., Yu, J. and Cleveland, T.E., 2006. Understanding the genetics of regulation of aflatoxin production and *Aspergillus flavus* development. Mycopathologia 162: 155-166.

Chen, W., Xing, D. and Chen, W., 2005. Rapid detection of *Aspergillus flavus* contamination in peanut with novel delayed luminescence spectra. Photochemistry and Photobiology 81: 1361-1365.

Dehghan, P., Zaini, F., Mahmoudi, M., Jebali, A., Kordbacheh, P. and Rezaei, S., 2008. Aflatoxin and sclerotia production in clinical isolates of *Aspergillus flavus* group. Iranian Journal of Public Health 37: 41-50.

Kabak, B., 2010. Prevention and management of mycotoxins in food and feed. In: Rai, M. (ed.). Mycotoxins in food, feed and bioweapons. Springer-Verlag, Berlin, Heidelberg, Germany, pp. 201-227.

Passone, M.A., Rosso, L.C., Ciancio, A. and Etcheverry, M., 2010. Detection and quantification of *Aspergillus* section *Flavi* spp. in stored peanuts by real-time PCR of nor-1 gene, and effects of storage conditions on aflatoxin production. International Journal of Food Microbiology 138: 276-281.

Pattron, D.D., 2006. *Aspergillus*, health implication and recommendations for public health food safety. International Journal of Food Safety 8: 19-23.

Pitt, J.I. and Hocking, A.D., 2009. Fungi and food spoilage. Springer, Dordrecht the Netherlands, 519 pp.

Putri, A.S.R., Retnowati, I. and Santiambarwati, 2003. Control of aflatoxigenic *Aspergillus flavus* in peanuts using non-aflatoxigenic *A. flavus*, *A. niger* and *Trichoderma harzianum*. BIOTROPIA 21: 32-44.

Scheidegger, K.A. and Payne, G.A., 2005. Unlocking the secrets behind secondary metabolism: a review of *Aspergillus flavus* from pathogenicity to functional genomics. In: Abbas, H.K. (ed.). Aflatoxin and food safety. Taylor and Francis, Boca Raton, FL, USA, pp. 137-165.

Schwartzburg, K.A., 2005. Gene expression analysis in *Aspergillus flavus* identified a gene, ahpA, involved in protection against organic peroxides. MSc thesis, North Carolina State University, Raleigh, NC, USA.

Shiferaw, B., Prasanna, B.M., Hellin, J. and Banziger, M., 2011. Crops that feed the world 6. Past successes and future challenges to the role played by maize in global food security. Food Security 3: 307-327.

Stroka, J. and Anklam, E., 2002. New strategies for the screening and determination of aflatoxins and the detection of aflatoxin-producing moulds in food and feed. Trends in Analytical Chemistry 21: 90-95.

Sun, X., Yan, L., Tang, Y. and Zhang, Y., 2012. A rapid and specific immunosensor for the detection of aflatoxigenic aspergilli. European Food Research & Technology 234: 1013-1021.

Surendranatha Reddy, E.C., Sudhakar, C. and Eswara Reddy, N.P., 2011. Aflatoxin contamination in groundnut induced by *Aspergillus flavus* type fungi: a critical review. International Journal of Applied Biology and Pharmaceutical Technology 2: 180-192.

Tran-Dinh, N., Kennedy, I., Bui, T. and Carter, D., 2009. Survey of Vietnamese peanuts, corn and soil for the presence of *Aspergillus flavus* and *Aspergillus parasiticus*. Mycopathologia 168: 257-268.

Turner, N.W., Subrahmanyam, S. and Piletsky, S.A., 2009. Analytical methods for determination of mycotoxins: a review. Analytica Chimica Acta 632: 168-180.

Van Egmond, H.P. and Jonker, M.A., 2004. Worldwide regulations for mycotoxins in food and feed in 2003. FAO food and nutrition paper. Food and Agriculture Organization of the United Nations, Rome, Italy, 165 pp.

Van Egmond, H.P., Schothorst, R.C. and Jonker, M.A., 2007. Regulations relating to mycotoxins in food: perspectives in a global and European context. Analytical and Bioanalytical Chemistry 389: 147-157.

Vietnam Ministry of Health, 2003. Thường quy kỹ thuật định danh nấm mốc *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus fumigatus* trong thực phẩm. Vietnam Ministry of Health, Phnom Penh, Vietnam. Available at: http://tinyurl.com/l4d5jh3.

Wang, J.S. and Groopman, J.D., 1999. DNA damage by mycotoxins. Mutation Research 424: 167-181.