DETECTION OF AFLATOXIN PRODUCING ASPERGILLUS FLAVUS ON PEANUT FROM THE NORTH OF VIETNAM BY MULTIPLEX PCR

XUAN HOI PHAM, QUANG HIEU DAM

Institute of Agricultural Genetics

ABSTRACT: Aflatoxins are among the most potent mutagenic, teratogenic, and carcinogenic natural compounds occurring in grains, foods and feeds. A numbers of studies have been focused on detection of aflatoxin producing aspergillus flavus. In this study, PCR approach - mediated method of detecting the aflatoxin-synthesizing genes in *Aspergilus* to identify Aflatoxin contamination degree in peanut from the North of Vietnam has been developed. A total of thirty strains of the *A. flavus* strains isolation on peanut from difference regions in the North of Vietnam and three *A. niger* strains, were subjected to PCR testing in an attempt to detect four genes, encoding for norsolorinic acid redutase (*nor-1*), versicolorin A dehydrogenase (*ver-1*), sterigmatocystin O-methyltransferase (*omt-1*) and a regulatory protein (*apa-2*), involved in aflatoxin biosynthesis. As a result from PCR reactions, nineteen strains of *Aspergiluss flavus* were shown to possess the four target DNA fragments, eight strains bearing less than the four target DNA fragments and others together with three *A. niger* strains were not contained any target DNA fragment suggesting that only strains of *Aspergiluss flavus* having PCR product of four target DNA fragments are aflatoxin producers. The nine strains of *Aspergiluss flavus* including seven strains containing four target DNA fragments, two strains did not contain any DNA fragment and 2 *A. niger* strains were subjected to multiplex PCR using the four pairs of primers complementing the coding region of the above genes. Interestingly, the four target DNA fragments were observed in all seven Aflatoxin producers, while others are not indicating that multiplex PCR is a suitable method for detection of Aflatoxin producing *Aspergiluss flavus*.

Aflatoxins are a family of polypetide secondary metabolites mainly produced by the important *Aspergillus flavus* group, such as *Aspergillus flavus, Aspergillus paraciticus* and *Aspergillus nomius*. They are strong hepatotoxin, carcinogens and found in various foods, feeds and grains like barley, corn, rice, beans, peanut and peanut products, wheat flour, spices, beer and are hazardous to human and animal health [18]. A lot of techniques and methods for aflatoxin determination have been developed. Conventional methods rely on microbiological techniques and immunological systems; both methods have drawbacks: the former is time consuming whereas the later is prone to aspecificity and may result in false positives [2]. An other widespread methods are thin-layer chromatography, high-performance liquid chromatography. However, these methods require facilities and mycological expertises [10, 17].

Studies on genomic function have been shown that, at least, more than 25 genes have been determined to involve in aflatoxin biosynthesis. These genes are clustered within a 70-kb DNA region in the chromosome aware involved in biosynthesis pathway, most of wich have been identified and their DNA sequences published [3]. Among these genes, *avfA* gene is involved in the conversion of averufin to versiconal hemiacetal acetate [20]. The *ver-1* gene codes for versicolorin A dehydrogenase, which converted the versicolin A to sterigmatocystin [11]. The *omtA* gene is involved in the conversion of sterigmatocystin to O-methylsterigmatosystin [18]. The *nor-1* gene codes for a reductase that converts norsolorinic acid to averanti [14] and *aflR* for A. flavus and *apa-2* for A. parasiticus is regulatory gene that activates the pathway genes [6, 15]. On the basic of the cloned and sequenced genes involved in aflatoxin biosynthesis, specific primers have been designed for PCR or multiplex PCR. In addition, genes of both

important aflatoxinogenic species, *A. flavus* as well as *A.paraciticus*, are very homologous, which mean that the same PCR systems can be used for detection of both species. Since, PCR approach like PCR, multiplex PCR, RT-PCR or real-time PCR is now applicable to detection of aflatoxin contermination in grains, foods and feeds [1, 2, 4, 5, 7, 9, 10, 12, 17].

Peanut is an important food crop that growth throught out of Vietnam. Peanut is commonly used in foods, feeds as well as an export product of Vietnamese. However, Peanut is seriously contamination of *A. flavus* induced aflatoxin. Since, the aim of this study is to develop PCR assays to detect aflatoxin contamination in peanut. However, application of PCR-based method for detection of potential aflatoxin-producing molds wil be applied for all grains, foods and feeds of Vietnam.

I. MATERIALS AND METHODS

1. Fungal strains, media and culture conditions

Thirty-three strains of *Aspergillus* spp., including thirty strains of *A. flavus* and three strains of *A.niger* were used in this study (listed in Table 2). All strains was isolated from three lots of peanut collected from the Northern areas (Dien Chau - Nghe An; Chi Linh - Hai Duong and Gia Lam - Hanoi) of Vietnam.

The fungal strains were isolated as follows: five or seven peanut grains from each lot were placed on potato dextro agar (PDA) plates and incubated for 36-48h at 28^0C in the dark. All point displaying olive-yellowish or green conidia and aspergillar-structured conidiophores at the thirth or fourth day of growth on PDA medium (modified with the addition of 100 µg/ml chlorotetracycline) were classified as *A. flavus* group. The strains were identified according to Samson's technique [8] and their morphological characteristics. The isolated strains were named with the shortcut of collected area following by a number indicating different strains in the same lot. Colonies classified as *A. flavus* were recovered and maintained on PDA medium at 28^0C in the dark by single spore method. It is stored for long-term in Czapek agar slants at -20^0C.

Table 1

Primers name and their sequence used in this study

Primers	Sequence
Fw Ver-1	5'-ATGTCGGATAATCACCGTTTAGATGGC-3'
Rv Ver-1	5'-CGAAAAGCGCCACCATCCACCCCAATG-3'
Fw Omt-1	5'-GGCCCGGTTCCTTGGCTCCTAAGC-3
Rv Omt-1	5'-CGCCCCAGTGAGACCCTTCCTCG-3
Fw Nor-1	5'-ACC GCT ACG CCG GCA CTC TCG GCA C-3'
Rv Nor-1	5'-GTT GGC CGC CAG CTT CGA CAC TCCG -3'
Fw Apa-2	5'-TAT CTC CCC CCG GGC ATC TCC CGG-3'
Rv Apa-2	5'-CCG TCA GAC AGC CAC TGG ACA CGG-3'

2. Genomic DNA extraction and PCR reaction

Total DNA was extracted from one day - old mycelia grown on PDA medium as described by Eric W. Boehm, 2004[3]. Four pairs of primers were employed to specifically amplify *apa-2*, *omt-1*, *ver-1* and *nor-1* genes (table 1).

PCR was performed in a volume of 20 µl reaction mix containing 1x Tag DNA polymerase buffer, 1 ng of total DNA as template, 25 $mmol^{-1}$ MgCl2, 0,25$mmol^{-1}$ of each dNTP, 0,5 µmol l^{-1} of each primers and 1U Taq polymerase. To improve yield and specificity of target genes in PCR amplifications, we include betaine and dimethyl sulfoxide (DMSO) - enhancing agents for PCR [14].

The thermocycle for specific PCR were adjusted to reach the optimal annealing temperature and optimal annealing, extension times depending on specific primer pairs of each gene.

The cycling parameters for multiplex PCR were a cycle of 5 min at 94°C, following 35 cycles of 1 min at 94°C, 1 min at 55°C and 1min at 72°C and an extension of 7 min. at 72°C.

The PCR products were analyzed by 1% agarose gel electrophoresis in 1X TBE, followed by ethidium bromide staining and UV light illumination.

II. RESULTS AND DISCUSSION

1. Isolation and characteization of *Aspergillus flavus* isolates and specific PCR with *ver-1*, *omt-1*, *apa-2* and *nor-1* primers

Thirty-three strains of *Aspergillus app*., including thirty strains of *A.flavus* and three strains of *A.niger* were isolated from many samples collecting from different areas on the North of Vietnam using Samson's technique in combining with their morphological characteristics. *A.niger* strains were nonaflatoxigenic and used as negative control.

Table 2

Presence of the target genes involved in aflatoxin biosynthesis of various molds belonging to *Aspergillus flavus* (HD, NA and GL) and three strains of *Aspergillus niger* (HDn, Naa and GLn)

Fungal strains	Target genes				Fungal strains	Target genes				Fungal strains	Target genes			
	Ver 1	Omt 1	Apa 2	Nor 1		Ver 1	Omt 1	Apa 2	Nor 1		Ver 1	Omt 1	Apa 2	Nor 1
HD3	+	+	+	+	**GL1**	+	+	+	+	**NA2**	+	+	+	+
HD4	+	+	+	+	**GL2**	+	+	+	+	**NA3**	+	+	-	+
HD5	+	+	+	+	**GL3**	+	+	-	-	**NA5**	+	+	-	-
HD6	+	-	-	-	**GL4**	+	+	+	+	**NA6**	-	-	-	-
HD7	-	-	-	-	**GL5**	+	+	+	+	**NA8**	+	+	-	+
HD8	-	-	-	-	**GL6**	+	+	+	+	**NA9**	+	+	+	+
HD9	+	+	+	+	**GL7**	+	+	+	+	**NA10**	+	+	+	+
HD10	+	-	+	+	**GL8**	+	-	+	+	**NA12**	+	+	+	+
HD12	+	+	+	+	**GL10**	+	-	+	+	**NA14**	+	+	+	+
HD14	+	+	+	+	**GL11**	+	+	+	+	**NA15**	+	+	+	+
HDn2	-	-	-	-	**GLn5**	-	-	-	-	**NAn5**	-	-	-	-

Note: (+). positive in detection; (-). negative in detection.

In order to annalyze *Aspergillus flavus* group for detection of aflatoxin producing *aspergillus* strains, The extracted DNAs of test strains were subjected to PCR detection of four specific genes involved in aflatoxin biosynthesis, including versicolorin A dehydrogenase (*ver-1*), stergmatocystin O-methyltransferase (*omt-1*), norsolorinic acid reductase (*nor-1*) and a regulatory protein that activates the pathway genes (*apa-2*) and the results are summarized in Table 2. Specific PCR reactions were performed with 35 cycles of three steps: 1 min at 94^{0}C denaturation; 1 min (at 55^{0}C for *ver-1* primer, at 58^{0}C for *apa-2* primer, at 56^{0}C for *omt-1* primer and at 55^{0}C for *nor-1* primer) for primers annealing and 1min at 72^{0}C for extension, followed by incubation at 72^{0}C for 7 min . As designed, PCR products of 896 bp, 1000 bp, 400 bp and 1400 bp are corresponding to *ver-1*, *omt-1*, nor-1 and *apa-2* respectively. Base on biosynthesis pathway of aflatoxin, we classify *Aspergiluss* strains having of four target genes as aflatoxigenic (Afla+strain) and Aspergiluss strains having of less than four target genes as nonaflatoxigenic (Afla-strain). Specific amplification of all four target gene products were obrerved only for genomic DNA extracted from nineteen strains of *Aspergiluss flavus* that are Afla+strain. The Afla-strains were separated into five groups on the basic of their PCR amplification pattern: three isolates displayed a three-banded pattern corresponding to *ver-1*, *apa-2* and *nor-1*; two isolates displayed a three-band pattern corresponding to *ver-1*, *omt-1* and *nor-1*; two isolates displayed a two-banded pattern

corresponding to *ver-1* and *omt-1*; one isolate (HD6) showed a one-band pattern (*ver-1*) and three isolates (HD7, HD8 and NA6) did not show any band. Three *Aspergillus niger* isolates did not display any band. As showed in Table 2, the *ver-1* gene was the most representative (82%) between the four aflatoxin structural assayed genes. Lower incidence were found for *nor-1*, *omt-1* and *apa-2* genes (73%, 70% and 67%).

Base on data in table 2, we further reconfirm specific amplification of the target genes using extracted DNA of among nineteen Afla+strains as templates and the results are presented in fig. 1.Figure 1a,b,c,d show the specific amplification of genes *ver-1*, *omt-1*, *nor-1* and *apa-2* respectively. All RCR reactions were amplified when extracted DNA of Afla+strains were used as templates, while no PCR reactions were amplified when extracted DNA of *A. nigers* were used as templates suggesting that PCR reactions are specificity and reproductive.

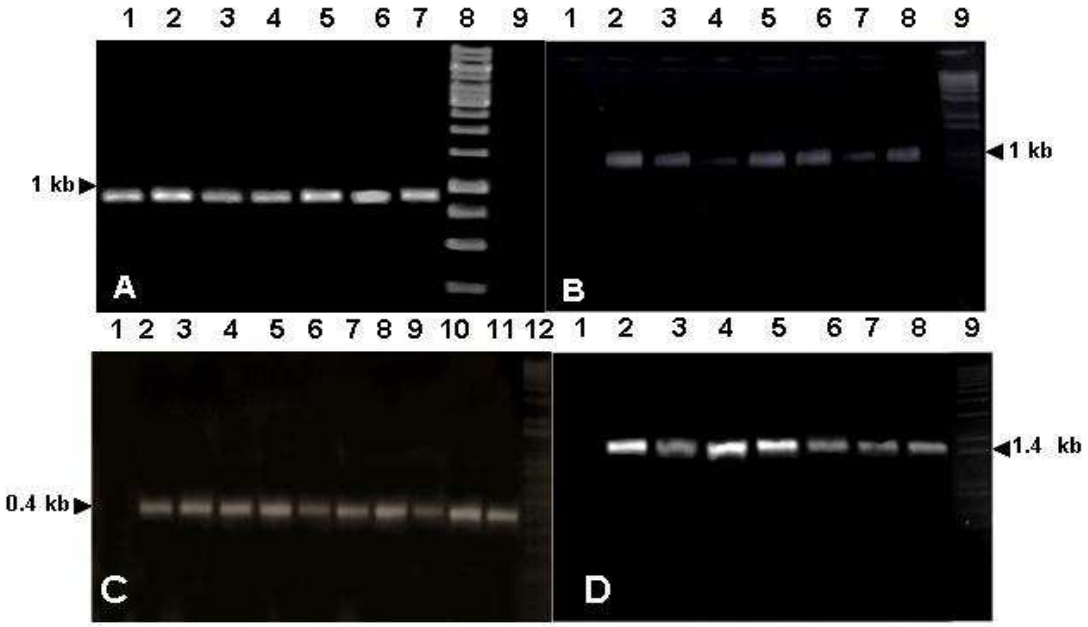

Figure 1. Agarose gel electrophoretic pattern of PCR products obtained from genomic DNA of Aspergillus flavus strains with the primer set specific for the ver-1 gene (a), the omt-1 gene (b), the nor-1 gene (c) and the apa-2 gene (d). Relevant fragment lengths are given in kb.

2. Multiplex PCR to detect *A. flavus*

In order to simplify PCR approach for detection of aflatoxigenic and nonaflatoxigenic *aspergillus* strains, a multiplex PCR was developed using the same set of primers employed in single gene PCR protocol. Nine strains of *A. flavus* from three lots including two strains from DH (HD8, HD9), three strains from NA (NA6, NA9, NA10) and 4 strains from GL (GL4, GL5, GL6, GL7) and two *A. niger* strains (NAn5, HDn2) as negative control were selected for multiplex PCR. Among nine *A. flavus* strains, seven strains are Afla+strains and other two are Afla-strains (table 2). Multiplex PCR reactions were performed with a cycle of 5 min at 94^0C, following 35 cycles of 1 min at 94^0C , 1 min at 55^0C and 1min at 72^0C and an extension of 7 min. at 72^0C. As expected, when genomic DNA of seven Afla+strains were used as a templates for multiplex PCR reactions, PCR products of four target DNA fragments corresponding for genes of norsolorinic acid reductase (*nor-1*), versicolorin A dehydrogenase (*ver-1*),sterigmatocystin O-methyltransferase (*omt-1*) and a regulation protein (*apa-2*), were observed (fig. 2, lane 2, 6, 7, 8, 9, 10, 11). The upper band was the *apa-2* fragment (~1400bp) and the following are *omt-1* (~ 1000bp), *ver-1*

(~900bp) and *nor-1*(~400bp) respectively. When genomic DNA of two *Afla-strains* and two *A. niger* strains were used as a templates for multiplex PCR reactions, none PCR products of these four target DNA fragments was detected (fig. 2, lane 1, 3, 4, 5). The data from multiplex PCR (fig. 2) are totally coincided to data from specific PCR (table 2), suggesting that multiplex PCR is selectable method for detaction of *aspergilus* spp. induced aflatoxin in foods, feeds and grains.

We now focuss on analysis of aflatoxin production by fluorescence developed on mycelium of the Afla+strains grown on CAM proved confirmatory as well as chromatography for the determination of afltoxin to support multiplex PCR approach for detection of aflatoxin producing *Aspergillus flavus*.

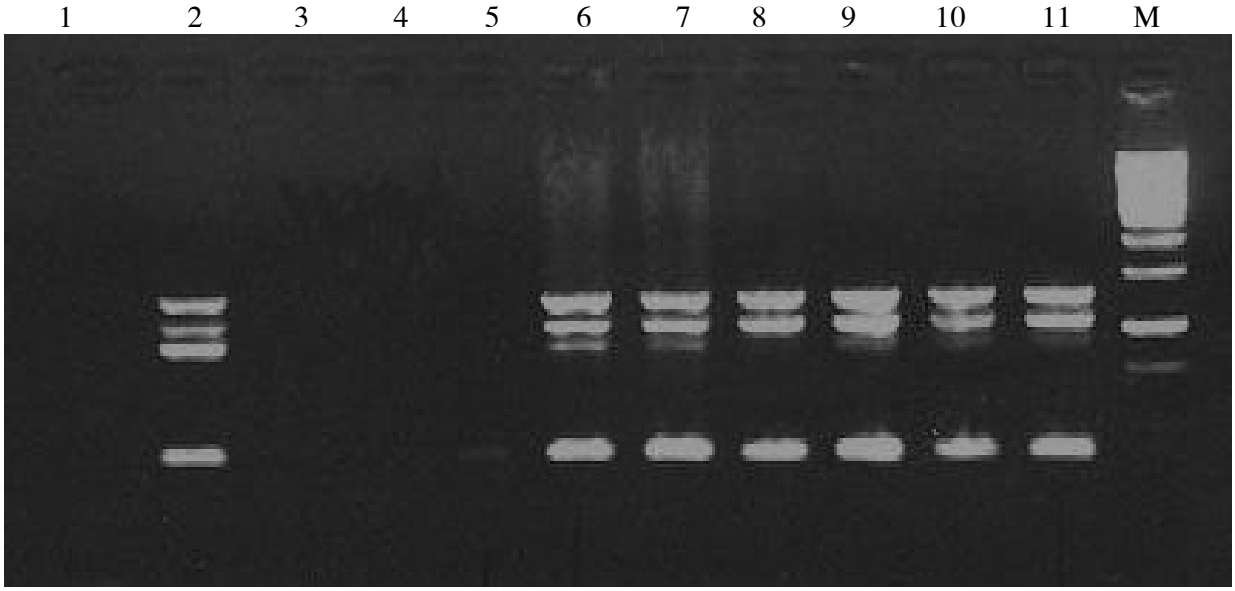

Figure 2. Expression of aflatoxin genes by Aspergillus flavus (wild strains) isolates. Transcripts of ver-1, omt-1, apa-2 and nor-1 were detected by multiplex PCR of total DNA from mycelium grown on PDA medium. Lane M: Ladder 1 kb; Lane 1: HDn2 as negative control; lane 2: HD9; lane 3: HD8; lane 4: NAn5 as negative control;lane 5: NA6; lane 6: NA9; lane 7: NA10; lane 8: GL4, lane 9: GL5, lane 10: GL6; lane 11: GL7.

REFERENCES

1. **Criseo G.**, **Bagnara A.** and **Bisignano G.**, 2001: Lett Appl Microbiol., 33: 291-295.
2. **Degola F. et al.**, 2007: Journal of Applied Microbiology, 103(2): 409-417.
3. **Giuseppe C.**, **Cosimo R.** and **Orazio R.**, 2008: International Journal of Food Microbiology, 125: 341-43.
4. **Manonmani, H. K.**, **Anand S.**, **Chandrashekar A.** and **Rati E. R.**, 2005: Process Biochem, 40: 2859-2864.
5. **Mayer Z.**, 2003b: Int. J. Food Microbiol., 82, 143-151.
6. **Perng-Kuang C.**, **Jeffery W. C.** and **Gary A. P.**, 1993: Applied and Environmental Microbiology, 59(10): 3273-3279.
7. **Ruey-Shyang C.**, **Jwu-Guh T.**, **Yu-Fen H.** and **Robin Y.-Y. C.**, 2002: Journal of food protection, 65(5): 840-44.
8. **Samson R. A. et al.**, 2004: Introduction to Food and Airborne Fungi, 7th edn. Wageningen: CBS.
9. Schabereiter-Gurtner C. et al., 2007: Journal of Clinical Microbiology, **45**(3): 906-914.
10. **Shapira R. et al.**, 1996: Appl. Environ. Microbiol., 62(9): 3270-3373.
11. **Skory C. D. et al.**, 1992: Appl. Environ. Microbiol., 58: 3527-3537.

12. **Somashekar D.** et al., 2004: Food Microbiol., 21: 809-813.
13. **Susan F. et al.**, 1998: Betaine and DMSO: Enhancing egents for PCR. Promega notes.
14. **Trail F.** et al., 1994: Appl. Environ. Microbiol., 60: 4078-4085.
15. **Watson A. J. et al.**, 1999: Appl. Environ. Microbiol., 65: 307-310.
16. **Wolfgang H. et al.**, 1997: Nucleic acids Research, 25(19): 3957-58.
17. **Zheng-You Y. et al.**, 2004: Journal of Food Protection, 67(11): 2622-26.
18. **Yu J. et al.**, 1993: Applied and Environmental Microbiology, 59(11): 3564-3571.
19. **Yu J. et al.**, 2000: Gene 248: 157-167.

PHÁT HIỆN NẤM *ASPERGILLUS FLAVUS* SINH ĐỘC TỐ AFLATOXIN TRÊN LẠC Ở MIỀN BẮC VIỆT NAM BẰNG PHẢN ỨNG MULTIPLEX PCR

PHẠM XUÂN HỘI, ĐÀM QUANG HIẾU

TÓM TẮT

Aflatoxin là một trong những chất gây đột biến, dị tật và ung thư nguy hiểm nhất chứa trong lương thực, thực phẩm và thức ăn. Rất nhiều nghiên cứu đang tập trung vào việc phát hiện nấm *Aspergillus flavus* sinh độc tố Aflatoxin. Trong nghiên cứu này, chúng tôi phát triển định hướng PCR thông qua việc phát hiện các gien sinh tổng hợp Aflatoxin trong nấm *Aspergillus* để khám phá trình trạng nhiễm Aflatoxin trên lạc ở miền bắc Việt Nam. Ba mươi chủng nấm *A. flavus* và ba chủng nấm *A. niger* (không sinh aflatoxin và sử dụng như đối chứng âm) phân lập trên lạc thu thập từ các tỉnh miền bắc Việt Nam như Nghệ An, Hải dương và Hà Nội được sử dụng trong phản ứng PCR nhằm phát hiện bốn gien (gien đích) *ver-1,omt-1, nor-1* và *apa-2,* tham gia vào quá trình sinh tổng hợp aflatoxin. Kết quả từ phản ứng PCR là mười chín chủng nấm *A. flavus* chứa cả bốn gien đích, tám chủng *A. flavus* chứa ít hơn bốn gen đích, ba chủng *A. flavus* và 3 chủng *A. niger* không chứa bất kỳ gen đích chứng tỏ chỉ có những chủng *Aspergiluss flavus* có cả bốn gien đích mới có khả năng sinh độc tố aflatoxin. Chín chủng *Aspergiluss flavus*, trong đó bảy chủng chứa cả bốn gien đích và hai chủng không chứa bất kỳ gien đích nào và hai chủng *A. niger* được sử dụng trong phản ứng PCR tổng hợp (multiplex PCR) sử dụng cả bốn cặp mồi đặc hiệu cho bốn gien đích. Kết quả phản ứng PCR tổng hợp cho thấy cả bốn gien đích chỉ được phát hiện trong bảy chủng nấm *A. flavus* sinh độc tố aflatoxin. Không một gien đích nào được phát hiện trong hai chủng nấm *A. flavus* không sinh độc tố a flatoxin và hai chủng nấm *A. niger*. Kết quả nghiên cứu chứng minh định hướng PCR tổng hợp là hoàn toàn phù hợp cho việc phát hiện nấm *A. flavus* sinh độc tố aflatoxin trong lương thực, thực phẩm và thức ăn.

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