Developing a PCR assay to detect *Aspergillus parasiticus*

- **Nguyen Huu Nhien**
- **Nguyen Thi Mai Tram**
  International University, VNU-HCM
- **Nguyen Thi Hue**
  University of Science, VNU-HCM

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ABSTRACT

Aspergillus parasiticus is one of the producers of aflatoxins in food. They may produce aflatoxin B and G which can lead to serious diseases in human. Aflatoxin is known as a type of carcinogens. People ingesting infected food even in a very low level for a long time can get cancer because of aflatoxin accumulation. Detection of aflatoxin or aflatoxin producers in foods is necessary to improve the life quality by decreasing the risk of getting diseases. Recently, the contemporary method to detect A. parasiticus is a morphological method but it still retains many limitations. In this study, a PCR based method was developed to provide a basic for develop a new method to detect A. parasiticus in food that overcame the disadvantages of the conventional morphological method. A specific set of primers was designed based on norB-cypA genes and successfully optimized for best amplification results at 62 ˚C. The sensitivity of the test was identified to be 0.005 ng/µL of target fungi DNA. A DNA isolation protocol was also optimized to ensure the success of the PCR assay, using SDS lysis, sand and thermal shock. The protocol from DNA isolation to PCR was successfully developed and provided a useful tool to improve the diagnosis of aflatoxins at an early stage and control all stages of food production. The success of this study is designing a pair of primers and a PCR assay which is specific for detection of A. parasiticus among other aspergilus species. This PCR assay can be used in the future for further development a PCR method for detection of A. parasiticus in food.

Key words: *Aspergillus parasiticus, aflatoxins, aflatoxin gene cluster, norB-cypA, PCR*

INTRODUCTION

Aspergillus species are among the most ubiquitously found mold fungi throughout the world which are of high importance in medicine, agriculture and biotechnology. Besides inducing direct pathogenesis, they also produce various types of toxic secondary metabolites, mycotoxins and cause non-contagious mycotoxicoses [1]. Out of all different types of mycotoxins, aflatoxins are the most potent natural carcinogens known, possessing hepatoxic and immunosuppressive properties which can cause acute liver damage, liver cirrhosis, tumor induction and teratogenesis [2]. They have been recognized as a possible human carcinogen by International Agency of Research on Cancer [3]. These mycotoxins are produced primarily by *Aspergillus parasiticus* and *A. flavus* which may invade agricultural products during plant growth, during harvest and

finally in storage, resulting in significant economic losses [4].

A. parasiticus, unlike *A. flavus* which only produces aflatoxin B, may produce both aflatoxins B and G. Discrimination between these two species and distinguishing them from closely related species is difficult when using conventional methods, which are mainly based on morphological or immunological features including culturing the fungus in suitable inducing media, extracting aflatoxins with other solvents, and monitoring their presence by chromatographic and ELISA techniques [5, 6]. These methods are time-consuming and require considerable performing expertise, which are their major drawbacks. Furthermore, the complexity in the presence of aflatoxinogenic and non-aflatoxinogenic *Aspergillus* species can cause false negative results in detection [7, 8].

The application of DNA-based techniques, particularly polymerase chain reaction (PCR), permits rapid, sensitive and specific detection that overcomes the disadvantages of conventional method, necessary to devise strategies to control and reduce fungal mass and toxin production at early and critical stages of the food chain. Up to date, there have been few studies on detection of *A. parasiticus* using molecular methods. RT-PCR with target genes aflD, aflO, aflP [9], PCR-RFLP with target gene aflR, multiplex-PCR with target genes nor-1, ver-1, omt-1 and apa-2 [10], real-time PCR with primers and probe designed on multicopy ITS2 rDNA target sequence have been used [11]. However, these methods were established to detect both *A. parasiticus* and *A. flavus* with the aim of monitoring aflatoxin-producing *Aspergillus* contamination of food and feedstuffs. A recent study on detection of only *A. parasiticus* using real-time PCR assay was performed by Sardinas N. in 2010 [11, 12] with primers designed on the multicopy internal transcribed region of the rDNA unit (ITS1-5.8S-ITS2 rDNA). This study was successful in accurately detecting and quantifying *A. parasiticus* at spore concentrations equal or higher than 10^6 spores/g. Nevertheless, this method needs to be performed in expensively equipped laboratories, which may increase the detection cost when applied in practice.

In this study, a cheap, accurate, sensitive and specific PCR assay is developed to detect *A. parasiticus.* The specificity of the assay was considerably improved when *norB-cypA* genes were used. These genes, which belong to the aflatoxin gene cluster, were shown to involve in biosynthesis of aflatoxins G [13-15]. Furthermore, they are highly variable among closely related species in *Aspergillus* genus, allowing successful detection of *A. parasiticus*. The possibility of this method will provide further information to develop a new method to predict mycotoxins profiles as well as to detect aflatoxin-producing species in food quality assurance labs in the future.

MATERIALS AND METHODS

Fungal isolates and culture conditions

Two different genera of filamentous fungi were used in this study (Table 1), including five *Aspergillus* species and one *Penicillium* species. Fungal strains were obtained from different Culture Collections in Vietnam or isolated from food. Morphological and genetic variations of *A. parasiticus* among intraspecies and extraspecies would be the important points to identify this species. Among these, both *A. parasiticus* and *A. flavus* belong to an important group of foodborne fungi which can produce aflatoxins. *A. oryzae* and *A. niger* are two fermented fungi which are "generally regarded as safe" (GRAS) by the FDA [16]; while *A. candidus* acts as a human pathogen causing invasive infection[17]. Finally, *P. aethiopicum* is an *Aspergillus*-related species. [18].

The isolates were maintained by regular subculturing on slant tubes containing Potato Dextrose Agar (PDA) (Merck, Germany) at 25 ˚C in the dark for 48 to 72 hours and then stored as spore suspension in sterile paraffin oil at 4˚C. Fungal strains were cultured for DNA extraction in Erlenmeyer flasks containing 50 mL of Malt Extract (ME) broth (Himedia, India) and incubated at 25 ˚C in an orbital shaker (120 rpm) for 48 to 72 hours. Mycelial mass was filtered through a filter paper, washed 3 times with NaCl 0.8 M and kept at -20 ˚C until DNA extraction.

Table 1. List of selected *Aspergillus* species and *Aspergillus*-related species

Species name	Accession number	Provider
Aspergillus parasiticus	VTCC-F-1130 VTCC-F-1132 VTCC-F-1159	Vietnam Type Culture Collection
Aspergillus flavus	VTCC-F-160	Vietnam Type Culture Collection
	VTCC-F-898	Vietnam Type Culture Collection
	AF.IV26.1	Pasteur Institute HCM city
	TN1	Isolated from maize – Quatest3
Aspergillus oryzae	VTCC-F-910 VTCC-F-912	Vietnam Type Culture Collection
Aspergillus niger	ATCC-16404	American Type Culture Collection
Aspergillus candidus		Isolated from food – Quatest3
Penicillium aethiopicum	TNTT	Quatest3

DNA extraction

DNA was extracted from mycelium following SDS method which was modified from Plaza's method [19]. The yield of the method was independently evaluated in all species shown in table 2. DNA concentrations were determined using a NanoDrop® 2000c spectrophotometer (Nanodrop Technologies, Wilmington, USA). The purity of the extractions was between 1.8 and 2.0. The DNA of the samples was diluted to 100 ng/µL.

PCR amplification

The assays were performed in a Veriti® 96-Well Fast Thermal Cycler (Applied Biosystems, USA). Amplification reactions were carried out in volumes of 10 µL containing 1.0 µL (100 ng) of template DNA, 1.0 µl of each primer (10 µM), 2.0 µL of 5X PCR buffer, 1.0 µL of $MgCl_2$ (25 mM), 0.8 µL of dNTPs (10 mM) and 1.0 µL of *Taq* DNA polymerase (5 U/µL) (Promega, USA).

PCR assay was performed using the thermal cycle of 5 minutes at 95 °C, then 35 cycles of 30 seconds at 95 °C, 30 second at annealing temperature, 30 seconds at 72 °C and finally 3 min at 72 °C. The annealing temperature was checked in the range of 58 -66 ^{o}C to get the best annealing temperature for further analysis.

PCR product was then analyzed in 2 % agarose ethidium bromide gel at 150 V in TBE 0.5 X buffer for 30 minutes and observed under

an ECX-20. M transilluminator (Vilber Lourmat, Germany).

RESULTS

Primer design

Primers were designed on the basis of sequence alignments of the *norB-cypA* genes, the farthest upstream portion of the aflatoxin biosynthesis gene cluster. This design, evaluated by Ehrlich [20, 21], was carried out on the completed sequence of aflatoxin pathway gene clusters of several strains from different origins, *Aspergillus parasiticus, A. flavus* AF13, *A. flavus* AF36, *A. flavus* AF70, *A. flavus* BN008R, *A. oryzae* RIB40, *A. normius* isolate AN13137 the information of which are available on NCBI website with GenBank accession numbers AY371490.1, AY510451.1, AY510455.1, AY510453.1, AY510452.1, AB196490.1, AY510454.1.

The primers APA1 (5' GGATTCGTGAGTGTCTTTAGGG 3') and APA2 (5' GGTAAATGCTCCGCACAGTC 3') fulfilled the requirements of specificity and efficacy required for *A. Parasiticus* identification. The amplicon for this set of designed primers is 343 bp. This amplicon also be checked based on the GenBank and the result shown that it is specific.

Gradient temperature PCR was performed to evaluate the primers annealing temperature to the gene specific to *A. paraciticus*. The gradient temperature PCR was performed with the range of temperature of 56-66 ^{o}C. The result showed that the target amplicon can be amplified easily at 56 ^{o}C, 58 ^{o}C, 60 ^{o}C, 62 ^{o}C and at 64 ^{o}C while at 66 ^{o}C there is no product for the PCR assay (Figure 1). To avoid extra-products at low temperature and lost of product at high temperature, 62 ^{o}C is selected at the best temperature for further experiments. Hence, the optimal temperature for the designed primer set was 62 ^{o}C.

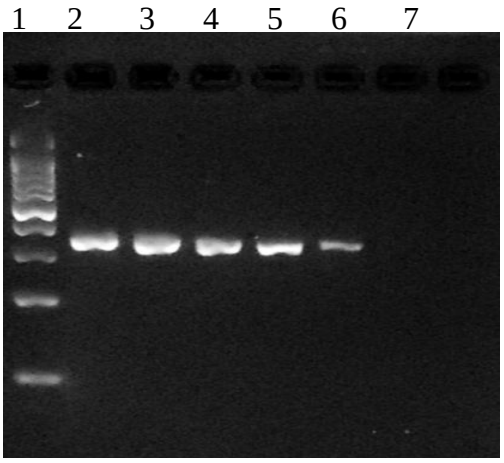

Figure 1. Optimizing annealing temperature for PCR assay amplifying the specific A. *paraciticus* target. Lane 1: 100bp –DNA molecular weight ladder. Lane 2- 7: annealing temperature at 56 ^{o}C, 58 ^{o}C, 60 ^{o}C, 62 ^{o}C, 64 ^{o}C and 66 ^{o}C. The selected annealing temperature is at 62 ^{o}C (lane 5).

The amplicon after be amplified is confirmed by sequencing. The target amplicon is sequenced by ABI 3130 (Namkhoa Company). The result showed that the amplified amplicon is the expected target which designed based on Genbank (Figure 2).

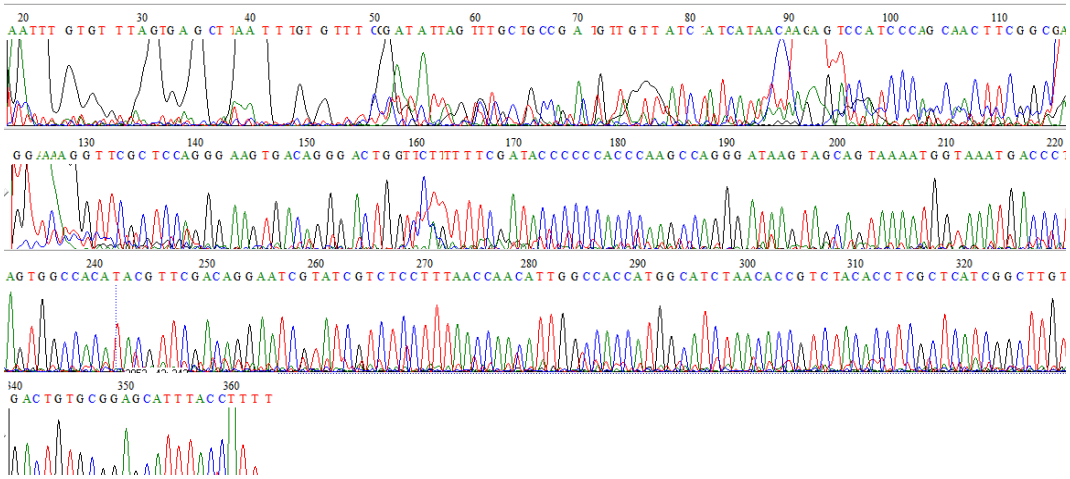

Figure 2. The DNA sequence of a specific *A.paraciticus* target of 343 bp.

Specificity testing

The specificity of the primer pairs APA1/APA2 for *A. parasiticus* was tested in PCR assays by using genomic DNA extracted from closely related species and genera that usually contaminate the same foods. The samples were prepared independently with DNA from individual strain at the concentration of 100 ng/µL. An additional sample containing DNA mixture of these molds with the same ratio in a concentration of 100 ng/µL was also used to test the capacity of the assay in detection of *A. parasiticus* contaminated with other species

The results shown in Figure 3 illustrated that the test could be used to detect the presence of *A. parasiticus*only in the tested samples (lane 2, 3, 4), while samples containing DNA solution of other species gave negative results (lane 5, 6, 7, 8, 9, 10, 11, 12, 13). The specificity of the test was also demonstrated when *A. parasiticus* was detected in the mixture of the DNA solution of all tested species (lane 1, Figure 3). This indicated that the test was specific for detecting *A. Parasiticus* in the presence of other species.

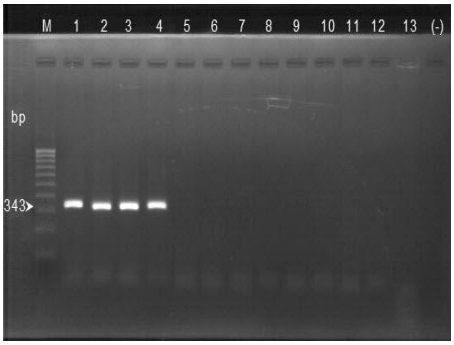

Figure 3. Gel electrophoresis of specificity testing. The target DNA band is at 343 bp. Lane 1: DNA mixture of selected species in table 1, lane 2: *A. parasiticus* VTCC-F-1130, lane 3: *A. parasiticus* VTCC-F-1132, lane 4: *A. parasiticus* VTCC-F-1159, lane 5: *A. Flavus* VTCC-F-160, lane 6: *A. Flavus* VTCC-F-898, lane 7: *A. Flavus* AF.IV26.1, lane 8: *A. Flavus* TN1, lane 9: *A. oryzae*VTCC-F-910, lane 10: *A. Oryzae* VTCC-F-912, lane 11: *A. niger* ATCC-16404, lane 12: *A. candidus*, lane 13: *P. aethiopicum*, M: 100-bp DNA molecular weight ladder, (-): negative control

Sensitivity testing

To assess the detection limit of the assay, two experiments were performed with a dilution series: 100 – 50 – 10 – 5 – 1 – 0.5 – 0.1 – 0.05 – 0.01 – 0.005 – 0.001 ng/µL made with DNA extracted from *Aspergillus parasiticus* VTCC-F-1132 and DNA mixture of these fungi (Table 1) with similar proportions of concentration.

The test was performed using the samples containing both DNA of *Aspergillus parasiticus* (VTCC-F-1132) only (Figure 4) and DNA mixture of all selected species (Table 1) at similar amount ratio (Figure 5). Figure 4 and Figure 5 showed a decrease in the intensity of bands from lane 1 to lane 11, corresponding to 100 ng to 0.001 ng in each microliter of PCR reaction, respectively. However, bands expressed in Figure 5 appeared significantly smeared when great amount of DNA was used (lane 1, 2, 3 and 4). This phenomenon might be caused by high productivity of amplified products. The bands in lane 11 of both Figure 4 and Figure 5 were considerably faint and difficult to be observed by naked eyes under UV luminescence. Thus, the previous band (lane 10), corresponding to 0.005 ng of DNA in each microliter, was selected as the limit of detection (LOD), i.e. LOD = 0.005 ng/µL.

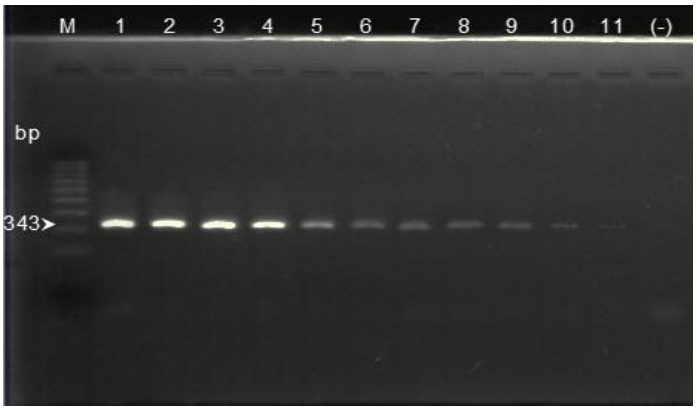

Figure 4. Gel electrophoresis of sensitivity testing on *Aspergillus parasiticus* VTCC-F-1132. The target DNA band is at 343bp. Lane 1: 100 ng, lane 2: 50 ng, lane 3: 10 ng, lane 4: 5 ng, lane 5: 1 ng, lane 6: 0.5, lane 7: 0.1, lane 8: 0.05, lane 9: 0.01, lane 10: 0.005, lane 11: 0.001. M: 100-bp DNA molecular weight ladder, (-): negative control.

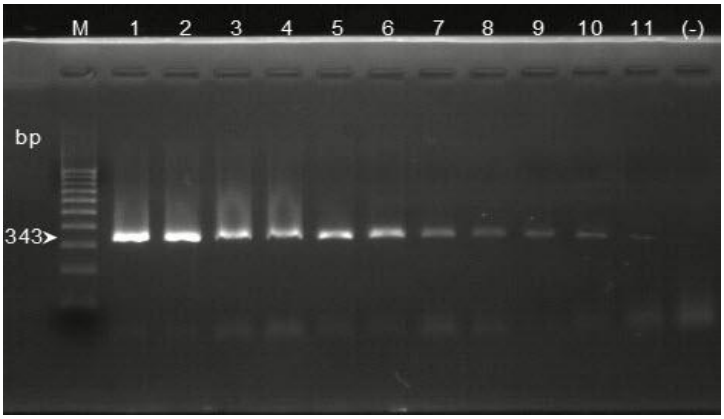

Figure 5. Gel electrophoresis of sensitivity testing on mixture of all selected species in Table 1. The target DNA band is at 343bp. Lane 1: 100 ng, lane 2: 50 ng, lane 3: 10 ng, lane 4: 5 ng, lane 5: 1 ng, lane 6: 0.5, lane 7: 0.1, lane 8: 0.05, lane 9: 0.01, lane 10: 0.005, lane 11: 0.001. M: 100-bp DNA molecular weight ladder, (-): negative control.

DISCUSSION

In this work, a highly specific method was developed to allow the detection of *Aspergillus parasiticus*, which would permit the prediction of whether aflatoxins type Gis present besides aflatoxins type B.

The sensitivity of PCR assay is dependent on the concentration of the target DNA input. The lowest detection limit of target DNA was estimated to be 0.005 ng/µL in comparison with 0.002 ng/µL in a similar study using real-time PCR method done by Sardinas [11]. However, with real-time PCR, the laboratories need to be equipped with expensive machine and costly chemicals. The validity of this assay was also confirmed when a mixture of target DNA and those of other related species with similar ratio were employed. A similar result to the one of pure target DNA was obtained.

The specificity of the assay was examined among a relatively diverse selection of strains and confirmed using a sample containing a DNA mixture of these strains. The use of the target sequence based on *norB-cypA* genes, which have shown to be an important factor in biosynthesis of aflatoxin type G in aflatoxin gene cluster, might enhance the specificity in the detection of *A. parasiticus* in comparison with other gene regions. Therefore, regarding its sensitivity, the PCR method would provide a better tool with high accuracy, rapidity and inexpensiveness for *A. parasiticus* as well as aflatoxins detection in comparison with the morphological methods.

However, to use this PCR assay for detection *A.paraciticus* in food it is necessary to perform more experiments to test the sensitivity of the test. Adding more controls such as internal control is necessary to avoid the fault results such as fault negative.

CONCLUSION

In this work, a PCR method using novel primers was successfully designed to detect the target genes *norB-cypA*, which is a part of the aflatoxin gene cluster. This PCR assay worked well at the annealing temperature of 62 ˚C, providing a specific, accurate and sensitive tool to detect *Aspergillus parasiticus* among others *Aspergillus* species. The limit of detection in this PCR assay was 0.005 ng/µL of pure genomic DNA. This assay and the designed primer pair can be used for continue developing a PCR method which can apply for detection of *A.paraciticus* in food in the future.

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Phát triển một phản ứng PCR phát hiện *Aspergillus parasiticus*

- **Nguyễn Hữu Nhiên**
- **Nguyễn Thị Mai Trâm**

Trường Đại học Quốc tế, ĐHQG-HCM

- **Nguyễn Thị Huệ**

Trường Đai học Khoa học Tự Nhiên, ĐHQG-HCM

TÓM TẮT

Aspergillus parasiticus là một trong các loại nấm mốc tiết độc tố aflatoxin trong thức ăn. Loại nấm mốc này có thể tiết ra aflatoxin B hay G dẫn tới bệnh nghiêm trọng ở người. Aflatoxin là một loại tác nhân gây ung thư. Người ăn phải thức ăn chứa dù chứa lượng nhỏ aflatoxin trong một khoảng thời gian dài có thể mắc phải ung thư do aflatoxin tích tụ. Do vậy, việc nhận biết aflatoxin và nhân tố tiết aflatoxin trong thực phẩm là cần thiết để tăng chất lượng cuộc sống bằng cách giảm nguy cơ mắc bệnh. Gần đây, phương pháp thông dụng để nhận biết A. parasiticus là phương pháp quan sát hình thái, tuy nhiên phương pháp này vẫn còn nhiều hạn chế. Đề tài này phát triển một phản ứng PCR truyền thống nhằm phát hiện A. parasiticus trong thực phẩm vượt qua những hạn chế của phương pháp hình thái hiện đang sử dụng. Một cặp mồi riêng biệt được thiết kế dựa trên genes norB-cypA đã được tối ưu hoá cho kết quả khuếch đại nhiều nhất ở 62 ^{o}C. Độ nhạy của phương pháp PCR này được xác định là có thể phát hiện DNA nấm mốc A. paraciticus ở nồng độ 0.005 ng/µL. Quy trình tách DNA cũng được tối ưu hoá để đảm bảo sự thành công cho phản ứng PCR bằng cách sử dụng ly giải SDS, cát và sốc nhiệt. Quy trình từ tách DNA đến PCR được phát triển có thể sẽ được ứng dụng để phát hiện A. parasiticus trong thực phẩm ở các giai đoạn khác nhau, tạo công cụ hữu dụng trong việc phát hiện aflatoxins ở giai đoạn đầu và kiểm soát tốt các giai đoạn sản xuất thực phẩm. Thành công của nghiên cứu này là thiết kế được cặp mồi và phản ứng PCR đặc hiệu có thể phân biệt A. parasiticus trong số các loài Aspergillus khác. Phản ứng PCR này có thể tiếp tục phát triển để có thể ứng dụng trong phát hiện A. parasiticus trong thực phẩm trong tương lai.

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