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Screening of Aflatoxigenic Fungi in Foods with Multiplex Polymerase Chain Reaction

Zhengyou Yang, Sungjo Kang, Junghyun Park, Duckhwa Chung

Division of Applied Life Science, Gyeongsang National University, Jinju, 660-701, Korea

Introduction

Aflatoxins are secondary metabolites commonly produced by the important food contaminating species *Aspergillus parasiticus* and *Aspergillus flavus* and potent liver carcinogens for a wide variety of animal species, including human. Various methods have been developed for detecting aflatoxin produced fungi in foods including chemical methods, morphological methods, immunological methods and bioassay methods. These conventional methods are time consuming, labor intensive and possesses the inherent possibility of false negative result. Rapid and more objective methods for detection of aflatoxigenic fungi in foods are needed for evaluating aflatoxin risks.

PCR assay is potentially more sensitive and rapid than conventional methods. The formation of aflatoxins involves over 20 enzymatic reaction, and most of the corresponding genes have been isolated and characterized. In the present study, we developed a multiplex PCR assay for the detection of aflatoxigenic fungi in foods with three genes, *avfA, omt-A*, and *ver-1*, coding for key enzymes in aflatoxin biosynthesis. Compare with DC-ELISA and TLC assay, we found that the multiplex PCR assay, is specificity and sensitivity and can be used for detection of aflatoxigenic fungi in foods.

Materials and Methods

Strains used in this study are cultured 4 days in PDB medium for preparation of mycelium and cultured 14 days in SLS medium for extracting aflatoxin. Fungal spore suspension were obtained by gently rolling a moistened, sterile cotton swab over the surface of the 7-10 days grown colonies and suspending in 30%(vol/vol) glycerol solution. Total DNA was extracted by a modification of a procedure described by Samarrai (2000) and monitored with ITS primers. The aflatoxins extracted from SLS medium are analyzed by DC-ELISA and TLC. The Conditions of multiple PCR were optimized for targeting *avfA*, *omt-A* and *ver-1* genes simultaneously in a single tube. For determination of the sensitivity of the multiplex PCR reaction, spore suspension of *Asp.parasticius* 15517 was diluted and inoculated 10^1, 10^2, 10^3, 10^4, 10^5, to 10^6 spores in 50 mL PDB respectively, and the extracted total DNA was used as templates in multiple PCR. To estimate the influence of the ratio between uniplex PCR and multiple PCR, the extracted DNA of *Asp. parasticius* 15517 was serially diluted as 1 pg, 10 pg, 100 pg, 1 ng, 10 ng, 100 ng and used respectively as templates in multiple PCR and in uniplex PCR. To test the

ability of the multiple PCR to distinguish aflatoxigenic molds from other molds found in foods, sterile barley were inoculated with 10^2 spores of *Asp. parasticius* 15517, 10^6 spores of *Fusarium. moniliforme* NRRL 13569, 10^6 spores of *Picillium. Expansum* and 10^6 spores of two nonaflatoxigenic *Aapergillus* species per gram.

Results and Discussion

The DNA extraction method, which used in this study, is effective for extracting fungi genomic DNA(Fig. 1).

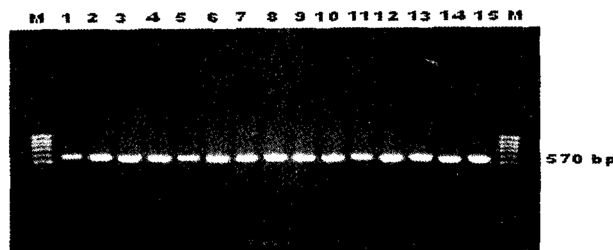

Fig. 1. Results of PCR with primer ITS specific to the fungal rDNA repeat unit.

Results of the specificity test for primers and correlated with detection of extracted fungi aflatoxin by TLC and DC-ELISA indicated that those primers designed and employed in this study were specific for the three targeted aflatoxin genes(Fig. 2). And good correlation was found between the multiplex PCR assay and the DC-ELISA and TLC. From the Fig. 2. D, we can see that the triplet band pattern of multiplex PCR was obtained only with aflatoxingenic fungi, and the multiplex PCR gave varying results with aflatoxin negative *Asp. flavus* strains, one strain showed no signal, other two strains the band for the *avfA* gene is missing. Other food related strains tested showed negative results for all the target genes.

The sensitivity of the uniplex PCR, using the serially diluted DNA of *Asp. parasiticus* ATCC 15517, was sufficient to amplify with 1 ng of temple DNA(Fig. 3. A); the lowest amount of DNA consistently amplifiable was 10 ng for the multiplex PCR(Fig. 3. B).

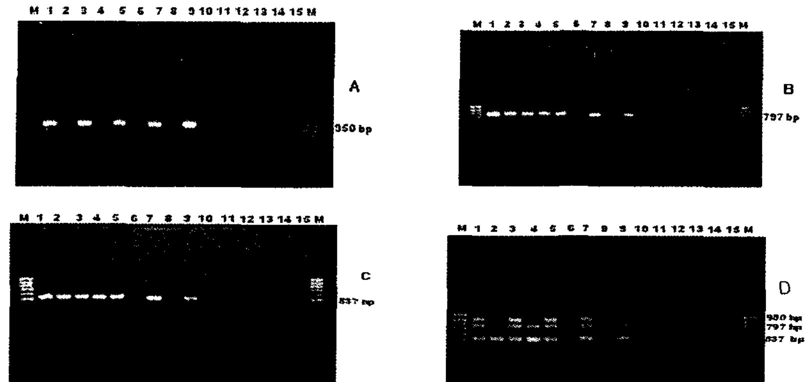

Fig. 2. Gel analysis of the uniplex PCR products with the primer pairs specific for the avfA gene(A), for omt-A gene(B), for the ver-1 gene(C) and the multiplex PCR products (D). M, molecular weight marker; Lane 1, *Asp. parasiticus* ATCC 24511 positive; Lane 2, *Asp.* IAM 2184 positive; Lane 3, *Asp. parasiticus* ATCC 15517 positive; Lane 4, *Asp. flavus*, IAM 2119 positive, Lane 5, *Asp. parasitics*, IAM 2703 positive; Lane 6, *Asp. flavus*, IAM 2044negative; Lane 7, *Asp. parasiticus* KGUF 1621 positive; Lane 8, *Asp.* KFRI 596 negative; Lane 9, *Asp. oryzae* KFRI 859 positive; Lane 10, *Asp. Niger* KFRI 988 negative; Lane 11, *Asp. niger* KFRI 990 negative; Lane 12, *Asp. oryzae* KFRI 998 negative; Lane 13, *Asp. usamii* KFRI 999 negative; Lane 14, *Fusarium. moniliforme* NRRL 13569 negative; Lane 15, *Penicillium. expansum* negative.

In both products, sensitivity of the amplification was decreased by 1 log in multiplex PCR compared to uniplex PCR. This suggested that competition among primer sets in multiplex PCR may contribute to a reduction in sensitivity of multiplex PCR.

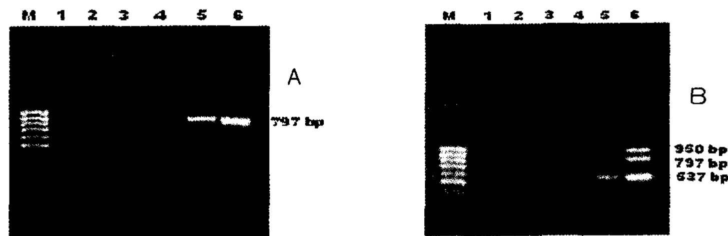

Fig. 3. Gel analysis of uniplex PCR(A) and multiple PCR(B) products serially diluted DNA from *Asp. parasiticus*ATCC 15517. Lane M, molecular weight marker; Lane 1, 1 pg negative; Lane 2, 10 pg negative; Lane 3, 100 pg negative; Lane 4, 1 ng, positive; Lane 5, 10 ng positive; Lane 6, 100 ng positive.

By the enrichment procedure, amplicons of multiplex PCR can be detected, even at the lowest spore level of *Asp. parasiticus* ATCC 15517(10^2 CFU/g), after 24 h of enrichment.

Inoculated with 10^2 spores of Asp.parasticius15517 in dried barley in enrichment media, we get the seem results. However, no DNA amplification was observed from barley inoculated with other fungi even at 10^6 spores/g.

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