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Detection of aflatoxinogenic fungi in figs by a PCR reaction

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Abstract

A PCR reaction was used to detect aflatoxinogenic *Aspergillus flavus* strains in contaminated figs. The reaction records the presence of three aflatoxin biosynthesis genes, namely the norsolorinic acid reductase (*nor-1*), versicolorin A dehydrogenase (*ver-1*) and sterigmatocystin-*o*-methyltransferase: (*omt-A*). The reaction gave a triplet pattern in the presence of DNA from *A. flavus* isolated from pure cultures. The reaction gave the same PCR products when pure fungal DNA was mixed with pure DNA isolated from figs, but the sensitivity was reduced by a factor of 10. The same set of bands was observed when isolated DNA from infected figs was used as template DNA but no signal was visible when DNA from uninfected figs was used as template. © 1997 Elsevier Science B.V.

Keywords: Aflatoxin; *Aspergillus flavus*; Multiplex PCR; Aflatoxin biosynthesis gene; Figs

1. Introduction

Aflatoxins are among the most toxic mycotoxins and are produced predominantly by *Aspergillus flavus* and *Aspergillus parasiticus* (Bennett and Papa, 1988). Aflatoxinogenic fungi are found in different food commodities including peanuts (Jelinek et al., 1989), spices (Llewellyn et al., 1992) and figs (Bullerman, 1979 Doster et al., 1996). Conventional methods to detect these fungi in foods include cultivation and taxonomic identification at the morphological level. This approach is, however, very time consuming and possesses the inherent possibility of misclassification. Rapid and more objective methods for the identification of aflatoxinogenic fungi in foods are needed for evaluating the microbiological risks of a given food.

PCR methods are frequently used for the detection of pathogenic or toxinogenic bacteria in foods (Olsen et al., 1995). For detection, unique sequences of these microorganisms are chosen as targets, which are predominantly toxin or pathogenicity genes. Equivalent genes for aflatoxinogenic fungi are the aflatoxin biosynthesis genes. Some of these genes have already been cloned and their sequences are known (Yu et al., 1995; Trail et al., 1995). It can be assumed that the aflatoxin biosynthesis genes are unique for the aflatoxin producing fungi. Starting from this hypothesis a multiplex PCR reaction for the detection of aflatoxinogenic fungi in vitro has been developed (Geisen, 1996). The reaction was

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targeted against three aflatoxin biosynthesis genes, namely norsolorinic acid reductase (*nor-1*), versicolorin A dehydrogenase (*ver-1*) and sterigmatocystin-*o*-methyltransferase (*omt-A*). These genes are nearly identical in *A. flavus* and *A. parasiticus* (Yu et al., 1995) which means that the same PCR system can be used for detection of both species. The functionality of the method was demonstrated in vitro. However it is known that food components can inhibit polymerase activity (Rossen et al., 1992). Here we describe the functionality of the method in a food system and estimate the reduction in sensitivity due to the presence of food components.

2. Materials and methods

2.1. Strains and culture conditions

Aspergillus flavus BFE84 was used as an aflatoxinogenic strain for all in vitro experiments, as well as for inoculation of fresh figs. *A. flavus* BFE84 produced only aflatoxin B_1 which was demonstrated by thin layer chromatography. *Fusarium poae* DSM62278 was used as control strain. Incubation was performed at 30°C for *A. flavus* and at 25°C for *F. poae*. Malt extract medium (Merck, Darmstadt) was used as standard medium. Figs were inoculated with the aflatoxinogenic *A. flavus* strain by cutting the surface of the figs and applying a spore suspension of *A. flavus* BFE84 onto the whole surface of the figs. They were then incubated at 30°C.

2.2. Isolation of fungal DNA

The isolation of DNA from fungal strains was performed according to a modified method originally described by Yelton et al. (1984). For that purpose 72 h to 96 h old mycelium was harvested from a submersed culture by filtration. The mycelium was transferred to a mortar and frozen in liquid nitrogen. The frozen mycelium was ground and then resuspended in lysis buffer (50 mM EDTA; 0.2% SDS; pH 8.5). This suspension was heated to 68°C for 15 min and then centrifuged for 15 min at $15000 \times g$. After centrifugation a volume of 7 ml of the supernatant was transferred to a new centrifuge tube and 1 ml of 4 M sodium acetate was added. This solution was placed on ice for 1 h and centrifuged for 15 min at $15000 \times g$. After centrifugation 6 ml of the supernatant was transferred to a fresh tube. The solution was phenol extracted and the isolated DNA was precipitated by the addition of 2.5 volumes of ethanol.

2.3. Isolation of DNA from figs

The isolation of DNA from figs was performed in the same way as the isolation of fungal DNA. Material from the surface of the figs was isolated, frozen in liquid nitrogen and ground in a mortar and the same preparation steps as with fungal DNA were carried out.

2.4. Determination of DNA concentration

The concentration of DNA was determined by a densitometer (Biorad, Model GS-670). A serial dilution of fungal chromosomal DNA was separated in an agarose gel, stained with ethidium bromide, photographed and scanned by the densitometer. The intensities of the bands were compared with a standard curve.

2.5. Polymerase chain reaction

The isolated chromosomal DNA was diluted to 2 ng/ml and used as template DNA for aflatoxin biosynthetic gene specific PCR reactions. A typical PCR reaction mixture contained: 5 μl template DNA, 5 μl *Taq* polymerase buffer, 1.25 μl primer (120 pmol/ml, each), 25 μl H_20 and 0.2 μl *Taq* polymerase (5 units/μl). Thirty PCR reaction cycles with the following temperature regime were performed: 95°C, 1 min; 65°C, 2 min; 72°C, 4 min. The sequences of the primers used were as follows: *nor-1*, 5′-ACCGCTACGCCGGCACTCTCGGCAC-3′; *nor-2*, 5′-GTTGGCCGCCAGCTTCGACAC-TCCG-3′, enclosing a fragment of 400 bp from nucleotide 501 to 900 of the *A. parasiticus nor-1* gene; *ver-1*, 5′-GCCGCAGGCCGCGGAGAAAG-TGGT-3′; *ver-2*, 5′-GGGGATATACTCCCGCGAC-ACAGCC-3′, enclosing a fragment of 537 nucleotides from nucleotide 623 to 1160 from the *A. parasiticus ver-1* gene; *omt-1*, 5′-GTG-GACGGACCTAGTCCGACATCAC-3′; *omt-2*, 5′-GTCGGCGCCACGCACTGGGTTGGGG-3′, en-

closing a fragment of 797 bp from nuclecotide 301 to 1098 from the *omt-A* gene of *A. parasiticus*. The lengths of the fragments are given according to the published sequences.

2.6. Determination of the sensitivity of the PCR reaction

To demonstrate the influence of food components on the sensitivity of the method the product yield of PCR reactions with pure fungal DNA was compared with PCR reactions with a mixture of fungal DNA and DNA from figs. For that purpose isolated DNA from *A. flavus* BFE84 was serially diluted. The concentration range of these dilutions covered four orders of magnitude with 5 pg as lowest and 50 ng as highest concentration. The diluted DNA was either used directly in a PCR reaction or 0.5 ng of isolated DNA from figs was added and these mixtures were used as templates in PCR reactions with the primer pair specific for the *omt-A* gene.

To estimate the influence of the ratio between fig and fungal DNA PCR reactions were carried out with mixtures of 20 pg of fungal DNA and increasing amounts of DNA from figs. The DNA from figs covered the concentration range between 5 pg and 5 ng per reaction.

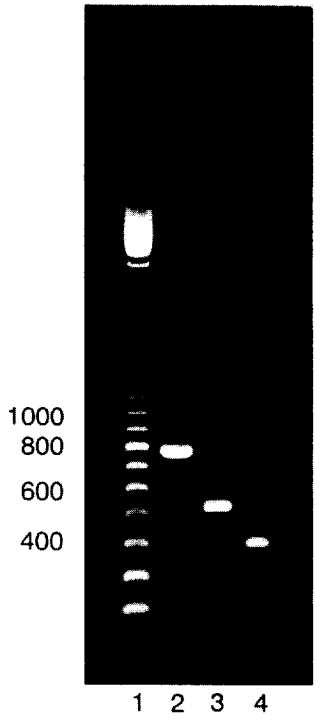

Fig. 1. Agarose gel electrophoresis of the PCR products of isolated DNA from *A. flavus* with the primer set specific for the *omt-A* gene (lane 2), the *ver-1* gene (lane 3) and the *nor-1* gene (lane 4). Lane 1, 100 bp ladder (Pharmacia); relevant fragment lengths are given in kb.

3. Results

To demonstrate the functionality of the PCR method, isolated DNA from the aflatoxinogenic strain *A. flavus* BFE84 was useful as template. Three PCR reactions, each specific for one of the aflatoxin biosynthesis target genes, were carried out. The simultaneous detection of three genes increases the specificity of the reaction. In the case of *A. flavus* the three expected amplicons became visible (Fig. 1). A more comprehensive analysis of the specificity of this reaction was published previously (Geisen, 1996).

It is known that food components can interfere with *Taq* polymerase, which can lead to false negative results in the worst case. Fig. 2 gives the result of an experiment which demonstrates the influence of food components on sensitivity of the reaction. There is a decrease in PCR product yield from the reaction with the highest template concentration to the reactions with the lower concentrations with both dilution series. A signal is visible in a wide concentration range between 50 ng and 25 pg template DNA per reaction with purified fungal DNA. The amount of 25 pg DNA per reaction was the minimal detection concentration with purified fungal DNA. According to the results of Keller et al. (1992) the genome size of *A. flavus* is approximately 35 Mb. Therefore it can be estimated that the minimum number of genome equivalents to give a positive reaction is about 0.7×10^5. When DNA from figs was present in the reaction mixture, the sensitivity of the reaction was decreased by a factor of 10. The minimal detection concentration was then 250 pg per reaction. However, a PCR product of the same size appeared, with no additional artifacts, indicating the precision of the reaction even under these conditions. The amount of DNA from figs was constant in these reactions. However, the ratio between fig and fungal DNA may have an influence

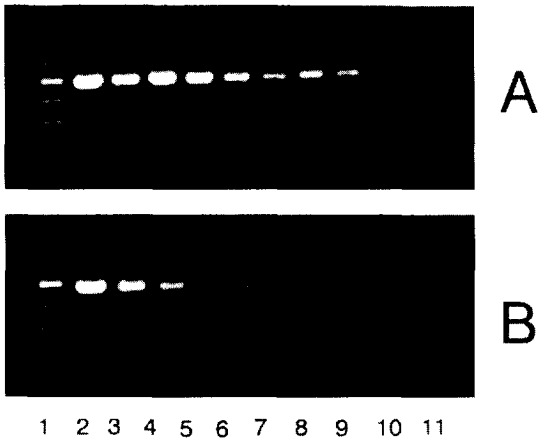

Fig. 2. Agarose gel electrophoresis of PCR products with the *omt-A* specific primer pair with decreasing amounts of pure *A. flavus* DNA as template DNA (A) and in the presence of a constant amount of DNA from figs (0.5 ng per reaction) (B). The concentrations of *A. flavus* DNA are the same in both reaction series. Lane 1, 100 bp ladder (Pharmacia); lane 2, 50 ng per reaction; lane 3, 5 ng per reaction; lane 4, 1 ng per reaction; lane 5, 0.5 ng per reaction; lane 6, 0.25 ng per reaction; lane 7, 0.1 ng per reaction; lane 8, 50 pg per reaction; lane 9, 25 pg per reaction; lane 10, 10 ng per reaction; lane 11, 5 pg per reaction.

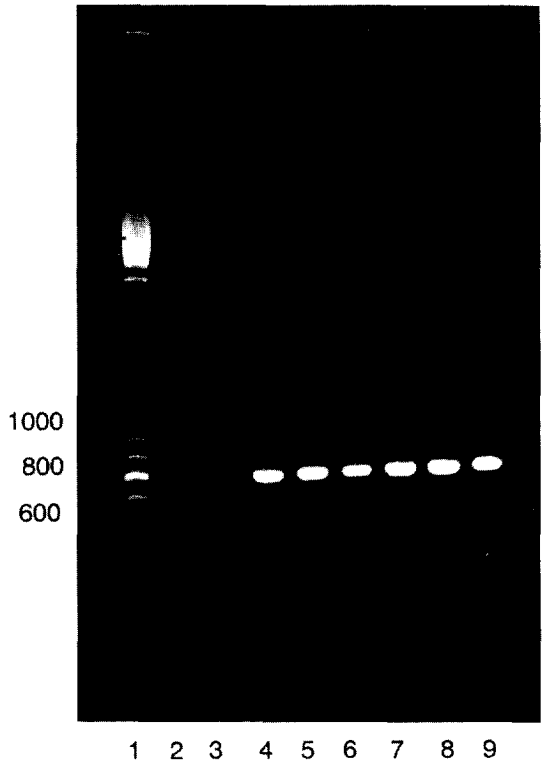

Fig. 3. Agarose gel electrophoresis of PCR products with the *omt-A* specific primer pair with a constant amount of *A. flavus* DNA (20 pg per reaction) and increasing amounts of DNA from figs. Lane 1, 100 bp ladder (Pharmacia); lane 2, 5 ng per reaction; lane 3, 2.5 ng per reaction; lane 4, 1 ng per reaction; lane 5, 0.7 ng per reaction, lane 6, 0.5 ng per reaction, lane 7, 0.25 μg per reaction; lane 8, 50 pg per reaction; lane 9, 5 pg per reaction.

on the sensitivity of this method. Fig. 3 shows the results of PCR reactions with a constant amount of fungal DNA but increasing amounts of DNA from figs. Only the two highest concentrations of DNA from figs inhibited the amplification reaction. All reactions with lower concentrations showed nearly identical band intensities.

To demonstrate the functionality of the method under practical conditions fresh figs were inoculated with *A. flavus* BFE84 and incubated at 30°C. Especially the surface area of the fruit tissue was used for the isolation of DNA. With this DNA PCR reactions specific for the three aflatoxin biosynthesis genes were performed. As a negative control, figs which were not infected with *A. flavus* were used. *A. flavus* DNA, and *A. flavus* DNA mixed with DNA from figs were used as positive controls. The result of this experiment is shown in Fig. 4. As expected, *A. flavus* DNA gave the three bands. The same result was achieved with the mixture of *A. flavus* DNA and DNA from figs. With DNA isolated from infected figs the same set of bands appeared. However in the reaction with the *omt-A* specific primer set two additional bands appeared and the PCR product specific for the *ver-1* gene was very weak. The reaction with the uninfected figs gave no signal at all. These results demonstrate the functionality of the PCR reaction with DNA isolated from the food system.

4. Discussion

A PCR method specific for the detection of aflatoxinogenic fungi has been applied in a food system. A PCR method for detection of aflatoxinogenic fungi has been described earlier (Geisen, 1996). Shapira et al. (1996) described a similar PCR approach for the detection of aflatoxinogenic fungi. They also used the genes *ver-1* and *omt-A* as targets,

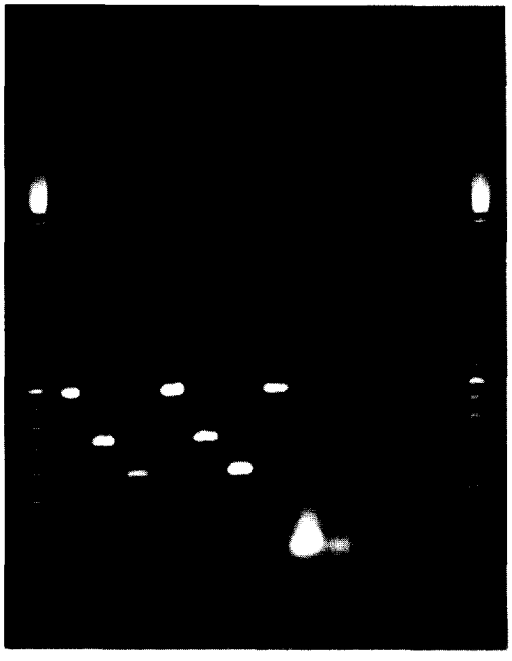

Fig. 4. Agarose gel electrophoresis of PCR products with the *omt-A* specific primer pair (lanes 2, 5, 8, 11), the *ver-1* specific primer pair (lanes 3, 6, 9, 12) and the *nor-1* specific primer pair (lanes 4, 7, 10, 13) with template DNA consisting of chromosomal DNA of *A. flavus* (lanes 2, 3, 4), DNA from *A. flavus* mixed with DNA prepared from uninfected figs (lanes 5, 6, 7), DNA prepared from infected figs (lanes 8, 9, 10) and with DNA prepared from uninfected figs (lanes 11, 12, 13). Lanes 1 and 14 100 bp ladder (Pharmacia).

but with the *apa-2* gene (now called the *aflR* gene) instead of the *nor-1* gene. They got clear results with purified DNA from *A. parasiticus*, but only weak signals with DNA from *A. flavus*. This difference from our results might be due to the different primer pairs used for amplification in both approaches. Diagnostic PCR reactions with food samples often give false negative results due to the presence of food components. Rossen et al. (1992) described that PCR is sensitive to complex food samples containing high amount of fat and proteins. According to our results, food samples containing high amounts of carbohydrates are inhibitory too. The sensitivity of the PCR reaction decreased when DNA from figs was present in the reaction mixture. In general, the higher the ratio between DNA from figs and fungal DNA, the greater the inhibition of the reaction. Widjojoadmodjo et al. (1991) found a 100 fold reduction in sensitivity of a PCR reaction specific for the detection of Salmonella when comparing the results from pure cultures with spiked samples of chicken meat. In our hands the sensitivity of the reaction is reduced by a factor of ten when DNA from figs was present in the reaction. According to our estimation, the minimal detectable concentration of genome equivalents in the reaction mixture is 0.7×10^5 in the case of pure fungal DNA; this is increased by a factor of ten when fig DNA is present in the sample. Given that vegetative cells are multinucleate, the minimum number of cells which can be detected by this method is lower than the number of genome equivalents. Shapira et al. (1996) used their PCR approach to detect aflatoxinogenic *A. parasiticus* strains in grain. They applied an enrichment procedure for amplification of template DNA prior to PCR. For this purpose the sample was dissolved in rich medium and incubated. The longer the incubation time the less fungal biomass could be detected. With an enrichment time of 24 h they were able to detect 10^2 spores initially present per gram of sample.

The problem of PCR inhibition by certain food components can perhaps be overcome by using adapted PCR or DNA preparation protocols. Kreutzinger et al. (1996) described a method for reducing inhibitory activity from environmental samples on PCR reactions. They used very diluted template DNA in a first PCR reaction, followed by a second reaction with nested PCR primers. Lantz et al. (1994) described another approach for the preparation of sample DNA in an attempt to detect pathogenic *Listeria monocytogenes* cells in soft cheese with the polymerase chain reaction. Functional template DNA could be prepared if an aqueous two phase sample preparation system was used. With this system the sample was homogenized in a mixture of two non-miscible aqueous solutions, which separate into two phases after homogenization. One phase consisted of a polyethylene glycol solution and the other of a dextran solution. This extraction system separated PCR-inhibiting compounds, which concentrated in the PEG phase from bacterial cells. When samples from the dextran phase were used for PCR the *L. monocytogenes* cells could be detected.

The PCR approach is a rapid assay for the presence of aflatoxinogenic fungi in certain food

systems. For routine analysis, the sample preparation protocol must be optimized carefully to avoid the occurrence of false negative reactions.

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