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Differentiation of aflatoxin-producing and non-producing strains of *Aspergillus flavus* group

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Aims: Three conventional methods and a multiplex PCR procedure with a set of four primers (Quadruplex-PCR) were used to differentiate between aflatoxin-producing and non-producing strains of the *Aspergillus flavus* group.
Methods and Results: By combining sets of primers for *aflR*, *nor-1*, *ver-1* and *omt-A* genes of the aflatoxin biosynthetic pathway, Quadruplex-PCR showed that aflatoxinogenic strains gave a quadruplet pattern, indicating the presence of all the genes involved in the aflatoxin biosynthetic pathway which encode for functional products. Non-aflatoxinogenic strains gave varying results with one, two, three or four banding patterns. A banding pattern in three non-aflatoxinogenic strains resulted in non-differentiation between these and aflatoxinogenic strains.
Conclusion and Significance and Impact of the Study: Because conventional methods are time-consuming, further studies are needed to develop a rapid and objective technique that permits complete differentiation between aflatoxin-producing and non-producing strains of the *A. flavus* group.

INTRODUCTION

Aspergillus flavus Link and *Aspergillus parasiticus* Speare are the most important aflatoxin-producing mould species. They can grow and release aflatoxins in several food and feed compounds (Ellis *et al.* 1991).

Aflatoxins are among the most carcinogenic natural compounds known (Eaton and Gallagher 1994), and are widespread in both industrial and developing countries (Moss 1991). Not all strains of *A. flavus* produce aflatoxins; several strains are non-toxinogenic because aflatoxin synthesis may become unstable in these fungi (Bennett and Christensen 1983).

Conventional methods used to differentiate between aflatoxin-producing and non-producing strains of the *A. flavus* group are based on culture on natural and/or artificial media which permits the release of aflatoxins (Davis *et al.* 1966; Lin and Dianese 1976; Wicklow *et al.* 1981; Filtenborg *et al.* 1983; Davis *et al.* 1987; Abarca *et al.* 1988; Bennett and Papa 1988). These methods are time-consuming, laborious and need the expertise of mycologists to avoid misidentification. Furthermore, they are not entirely reliable in distinguishing potential aflatoxin-producing strains of the *A. flavus* group from non-toxinogenic strains.

Recently, biomolecular techniques have been developed to distinguish and identify aflatoxinogenic strains of *A. flavus* and *A. parasiticus* from other food-borne fungi (Geisen 1996; Shapira *et al.* 1996; Färber *et al.* 1997). Geisen (1996) used multiplex PCR with three sets of primers specific for three structural genes of the aflatoxin biosynthetic pathway (*nor-1*, *ver-1* and *omt-A*), and was able to differentiate *A. flavus* and *A. parasiticus* from other food-borne fungi, but not aflatoxin-producing and non-producing strains of the same species. Shapira *et al.* (1996) used standard PCR to detect aflatoxinogenic strains in grains. The authors used aflatoxin-producing strains and carried out monomeric PCRs with three different sets of primers for *apa-2* (actually named *aflR* (Woloshuk *et al.* 1994)), *omt-1* and *ver-1* genes of the aflatoxin biosynthetic pathway. They discriminated aflatoxinogenic strains from other moulds commonly inhabiting stored grains. Färber *et al.* (1997) detected aflatoxin-producing strains of *A. flavus* in contaminated figs by performing a monomeric PCR with the same sets of primer used by Geisen (1996). These techniques are more rapid and objective than conventional methods for distinguishing and

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identifying potential aflatoxinogenic strains of *A. flavus* and *A. parasiticus* from other food-borne fungi. However, none of these methods has yet been able to reliably differentiate between aflatoxinogenic and non-aflatoxinogenic strains of the *A. flavus* group.

In the present study, three conventional methods and a new multiplex PCR procedure with four sets of primers (Quadruplex PCR) were used to differentiate between aflatoxin-producing and non-producing strains of the *A. flavus* group.

MATERIALS AND METHODS

Organisms

Eleven *A. flavus* (MAM 002, 076, 077, 083, 084, 085, 086, 087, 088, 092, 096) and one *A. parasiticus* (MAM, 090), used throughout this investigation, were obtained from the culture collection of the Institute of Microbiology, Faculty of Science, University of Messina, Italy. The strains were isolated from herbal drugs and food commodities for human consumption.

Inoculum preparation

The isolates were kept in physiological salt solution containing 0·01% Tween 80 (PST); they were inoculated onto Potato Dextrose Agar (PDA, Difco) plates and incubated at 30°C for 7 days. After this period, the spores were recovered by washing the PDA surface with sterile PST. The harvested spores were enumerated using a Thoma chamber and diluted to approximately 10^8 ml^{-1}. This spore suspension was used as an inoculum.

Determination of aflatoxin production

For the determination of aflatoxin production, three different media were used: Malt Glucose Agar (MGA: malt extract agar (MEA, Merck), 5% glucose), modified Coconut Agar medium (CAM: Lin and Dianese 1976; Davis *et al.* 1987) and Yeast Extract Sucrose (YES: 2% yeast extract and 20% sucrose; Davis *et al.* 1966).

The inocula in MGA and CAM were prepared as follows. Spore suspension (10 μl) of each strain was inoculated in duplicate at the centre of Petri dishes containing 20 ml of the media. After inoculation, MGA and CAM plates were incubated upside down at 26°C for 5 and 7 days, respectively. The inoculum in YES medium was by inoculating 0·1 ml of each spore suspension, in duplicate, onto 50 ml of the medium and then incubating at 28°C as stationary cultures for 14 days.

For the strains grown in MGA, aflatoxins were extracted by the following protocol. One colony of each strain was transferred to an Eppendorf tube and 500 μl chloroform were added. The mixture was shaken for 20 min at room temperature, the mycelium was discarded and the chloroform extract was evaporated to dryness in a Speed Vac Concentrator. The residue was re-dissolved in 10 μl chloroform and applied onto a TLC plate (20 × 20 cm, Merck, Darmstadt, Germany).

For the strains grown in CAM, the procedure for aflatoxin extraction was as follows. Colonies grown on one plate, together with the medium, were finely crumbled (or cut), put into a separating funnel and extracted twice with 15–20 ml chloroform. Samples were shaken for 15 min at 37°C on a horizontal shaker (Gerhardt, Bonn, Germany) at 120 strokes min^{-1}. The suspensions were filtered through filter paper (Whatman no. 1) and then dehydrated in a column containing glass wool and 2·5 g anhydrous sodium sulphate. The samples were then evaporated nearly to dryness in a Rotavapor in N_2 atmosphere. The residue of each sample was re-dissolved in 500 μl chloroform and 10 μl of this solution were applied to TLC plates.

For the strains grown in YES medium, aflatoxin extraction was carried out as follows. After incubation, the entire culture was blended and extracted with 50 ml chloroform. The mycelial mat was separated from the broth by filtering through Whatman no. 1 filter paper. The filtrate was transferred to a separating funnel and the chloroform layer filtered through anhydrous sodium sulphate. The extraction procedure was repeated twice using 50 ml chloroform each time. Extracts were combined and evaporated to dryness in a Rotavapor in N_2 atmosphere. The residue of each sample was re-dissolved in 1 ml chloroform and 10 μl of this solution were applied to TLC plates.

The solvents toluol (50 volumes), ethylacetate (30 volumes) and acetic acid (4 volumes) were used. Pure aflatoxin (Makor Chemicals) was used as standard. The spots were visualized under u.v. light (366 nm).

Isolation of fungal DNA

The isolation of DNA from fungal strains was performed according to the method described by Yelton *et al.* (1984), with some modification.

The strains were grown for 72 h under conditions of continuous shaking (180 rev min^{-1}) in Malt Extract Broth (MEB; Merck). The mycelium was then harvested by filtration, transferred to a mortar, frozen in liquid N_2 and ground to a powder. The powder was resuspended in Lysis buffer (50 mmol l^{-1} EDTA, 0·2% SDS pH 8·5) and immediately heated at 68°C for 15 min. After centrifugation for 15 min at 15 000 *g*, a volume of 7–10 ml of the supernatant fluid was transferred to a new centrifuge tube and 1 ml 4 mol l^{-1} sodium acetate was added. This solution was placed on ice for 1 h and centrifuged for 15 min at

15 000 *g*. After centrifugation, the supernatant fluid 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.

Quadruplex PCR reaction

PCR reaction was performed according to Färber *et al.* (1997) with some minor modifications. The isolated chromosomal DNA was diluted to 2 μg ml^{-1} and used as template DNA for aflatoxin biosynthetic gene specific multiplex PCR reaction. A typical PCR mixture contained: 5 μl DNA template, 5 μl Taq polymerase buffer, 1·25 μl primer (120 pmol ml^{-1} each), 25 μl H_2O and 0·1 μl Taq polymerase (5 U μl^{-1}). A total of 35 PCR cycles with the following temperature regimen was performed: 95°C, 1 min; 65°C, 30 s; 72°C, 30 s for the first cycle; and 94°C, 30 s; 65°C, 30 s; 72°C, 30 s for the next 34 cycles. The sequences of the primers used were as follows: nor1, 5′-ACC-GCTACGCCGGCACTCTCGGCAC-3′, nor2, 5′-GTT-GGCCGCCAGCTTCGACACTCCG-3′ enclosing a fragment of 400 bp from nucleotide 501–900 of the *A. parasiticus nor-1* gene; ver1, 5′-GCCGCAGGCCGCGGA-GAAAGTGGT-3′, ver2, 5′-GGGGATATACTCCCGC-GACACAGCC-3′, enclosing a fragment of 537 bp from nucleotide 623–1160 of the *A. parasiticus ver-1* gene; omt1, 5′-GTGGACGGACCTAGTCCGACATCAC-3′, omt2, 5′-GTC-GGCGCCACGCACTGGGTTGGGG-3′, enclosing a fragment of 797 bp from nucleotide 301–1098 of the *A. parasiticus omt-A* gene; aflR1, 5′-TATCTCCCCCCGG-GCATCTCCCGG-3′, aflR2, 5′-CCGTCAGACAGCCA-CTGGACA-CGG-3′, enclosing a fragment of 1032 bp from nucleotide 450–1482 of the *A. parasiticus aflR* gene.

All experiments were repeated twice.

RESULTS AND DISCUSSION

The results obtained by conventional and molecular methods are shown in Table 1, Table 2 and Fig. 1.

The determination of aflatoxin production is shown in Table 1. Of 12 strains, nine (MAM 076, 077, 083, 085, 086, 087, 088, 092 and 002) were non-aflatoxin producers and only three (MAM 084, 090 and 096) were aflatoxin producers. *Aspergillus flavus* aflatoxin-producing strains released only aflatoxins of B group, while *A. parasiticus* strain also produced the G group. Aflatoxin production was similar in MGA and CAM for all the strains examined. *Aspergillus flavus* MAM 084 and *A. parasiticus* MAM 090 failed to produce aflatoxin B2 and G2, respectively, on YES medium. *Aspergillus flavus* 096 produced only aflatoxin B2. TLC analysis has shown a clear differentiation between aflatoxin-producing and non-producing strains of *A. flavus* and *A. parasiticus*.

Multiplex PCR was developed using four sets of primer for *aflR*, *omt-A*, *ver-1* and *nor-1* genes, enclosed in the aflatoxin biosynthetic pathway. Figure 1 and Table 2 show the results obtained for the strains examined. Bands of the fragments of *aflR*, *omt-A*, *ver-1* and *nor-1* genes can be visualized at 1032, 797, 537 and 400 bp, respectively (Fig. 1).

All aflatoxinogenic strains, M084 (lane 4, Fig. 1), M090 (lane 9, Fig. 1) and M096 (lane 11, Fig. 1), showed a quadruplet pattern, indicating the presence of the four genes of the aflatoxin biosynthetic pathway, whereas non-aflatoxinogenic strains presented varying patterns.

Table 2 compares the results obtained by Quadruplex PCR and conventional methods. There is a correlation for aflatoxin-producing strains (MAM 084, 090 and 096) where, as regards aflatoxin production, a complete pattern with four bands obtained in Quadruplex PCR is always related to a positive response obtained by conventional methods. For

Table 1 Aflatoxin production on cultural media (Yeast Extract Sucrose medium-YES, Malt Glucose Agar-MGA and Coconut Agar Medium-CAM)

	Afla B_1			Afla B_2			Afla G_1			Afla G_2		
Strains	YES	MGA	CAM	YES	MGA	CAM	YES	MGA	CAM	YES	MGA	CAM
A. flavus MAM 076	–	–	–	–	–	–	–	–	–	–	–	–
A. flavus MAM 077	–	–	–	–	–	–	–	–	–	–	–	–
A. flavus MAM 083	–	–	–	–	–	–	–	–	–	–	–	–
A. flavus MAM 084	+	+	+	–	+	+	–	–	–	–	–	–
A. flavus MAM 085	–	–	–	–	–	–	–	–	–	–	–	–
A. flavus MAM 086	–	–	–	–	–	–	–	–	–	–	–	–
A. flavus MAM 087	–	–	–	–	–	–	–	–	–	–	–	–
A. flavus MAM 088	–	–	–	–	–	–	–	–	–	–	–	–
A. parasiticus MAM 090	+	+	+	+	+	+	+	+	+	–	+	+
A. flavus MAM 092	–	–	–	–	–	–	–	–	–	–	–	–
A. flavus MAM 096*	–	–	–	+	+	+	–	–	–	–	–	–
A. flavus MAM 002	–	–	–	–	–	–	–	–	–	–	–	–

–: negative; +: positive.

*Only aflatoxin B2 was produced by the strain MAM 096.

Table 2 Comparison between the results obtained by Quadruplex PCR and conventional methods

Strains	*aflR*	*omt-A*	*ver-1*	*nor-1*	Aflatoxin production
A. flavus MAM 076	+ –	+ –	+	+	Negative
A. flavus MAM 077	–	–	+	–	Negative
A. flavus MAM 083	–	–/+	+	–	Negative
A. flavus MAM 084	+	+ –	+	+	Positive
A. flavus MAM 085	–	+ –	+	–	Negative
A. flavus MAM 086	+	+	–	+	Negative
A. flavus MAM 087	–/+	–	–/+	+	Negative
A. flavus MAM 088	+	+	–	+	Negative
A. parasiticus MAM 090	+	+	+	+	Positive
A. flavus MAM 092	+	+	+	+	Negative
A. flavus MAM 096	+	–/+	+	+	Positive
A. flavus MAM 002	+	+ –	+	+	Negative

+: Normal signal; ± : weak signal; –/+: very weak signal; –: no signal.

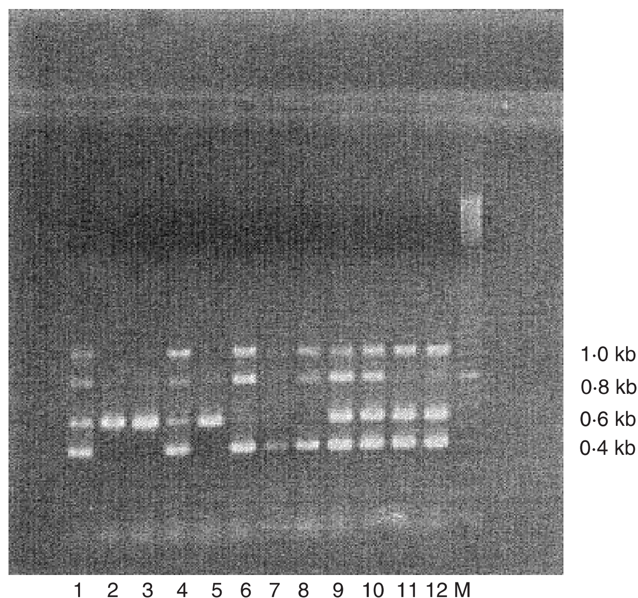

Fig. 1 Agarose gel electrophoresis of Quadruplex PCR products. Lane M: molecular size markers (Pharmacia 100 bp ladder); lane 1: *Aspergillus flavus* MAM076; lane 2: *A. flavus* MAM077; lane 3: *A. flavus* MAM083; lane 4: *A. flavus* MAM084; lane 5: *A. flavus* MAM085; lane 6: *A. flavus* MAM086; lane 7: *A. flavus* MAM087; lane 8: *A. flavus* MAM088; lane 9: *A. parasiticus* MAM090; lane 10: *A. flavus* MAM092; lane 11: *A. flavus* MAM096; lane 12: *A. flavus* MAM002

non-aflatoxin-producing strains there is no correlation between the results obtained by Quadruplex PCR and conventional methods. The presence of a complete pattern in the non-aflatoxinogenic strains shows that it is not a sufficient marker for differentiation between aflatoxinogenic and some non-aflatoxinogenic strains. Lack of aflatoxin production apparently need not only be related to an incomplete pattern obtained in Quadruplex PCR. This suggests that different types of mutations may have inactivated the aflatoxin biosynthetic pathway of these strains. This was also observed by Geisen in a previous study (Geisen 1996).

In the present study an additional set of primers specific for the *aflR* gene was also used. This gene plays an important role in the aflatoxin biosynthetic pathway by regulating the activity of other structural genes such as *omt-A*, *ver-1* and *nor-1* (Woloshuk *et al.* 1994; Chang *et al.* 1999). Multiplex PCR with four sets of primers has permitted the clear detection of aflatoxinogenic strains of *A. flavus* and *A. parasiticus* by the presence of a complete pattern with four bands, thus indicating the presence of all the genes examined which encode for functional products. The presence of a quadruplet pattern for some non-aflatoxinogenic strains of *A. flavus* indicates that the lack of aflatoxin production could also be due to simple mutations (substitution of some bases) which lead to the formation of non-functional products.

Aflatoxin production is controlled by a mechanism of regulation of structural gene transcription in which *aflR* plays a very important role. Liu and Chu (1998) showed, by a time-consuming and very laborious hybridization technique, that AFLR, a product of the *aflR* gene, regulates the expression of the *omt-A* gene, a structural gene enclosed in the aflatoxin biosynthetic pathway. They found AFLR in all the non-aflatoxinogenic strains in *Aspergillus* section *flavi* examined. However, *omt-A* was not expressed, even though it was present in all the strains.

Quadruplex PCR has proved to be a very precise and rapid biomolecular technique for detecting aflatoxinogenic strains of *A. flavus* and *A. parasiticus*, but it does not always permit discrimination between them and non-aflatoxin-producing strains. Indeed, the presence in some non-aflatoxinogenic strains of the complete pattern with four bands did not

distinguish these strains from the aflatoxinogenic strains in which a quadruplet pattern is anyway always present.

Conventional methods using cultural media for aflatoxin production distinguished perfectly between aflatoxin-producing and non-producing strains in the present study. Unfortunately, these methods are time-consuming and above all, as reported in the literature, they can fail to detect of some aflatoxin-producing strains because instability of aflatoxin production may occur in certain toxigenic strains growing in culture media (Abarca *et al*. 1988; Lemke *et al*. 1989). Further studies are needed to develop a rapid and more objective technique that permits clear differentiation between aflatoxin- and non-aflatoxin-producing strains of *A. flavus* and *A. parasiticus*.

Doohan *et al*. (1999) developed a RT-PCR assay to quantify expression of the *tri5* gene involved in the trichothecene biosynthetic pathway of the *Fusarium* species. They observed that *tri5*-PCR could provide a screening tool for the detection of tricothecene-producing *Fusarium* sp. Sweeney *et al*. (2000) used a RT-PCR for monitoring aflatoxin production in *A. parasiticus*. The authors suggested that this assay has the potential of being able to detect a number of different mRNA transcripts from aflatoxin genes when the producing fungus is cultured under a variety of different physiological conditions affecting aflatoxin biosynthesis.

It would be interesting to use the RT-PCR biomolecular technique, analogous to that made by Doohan *et al*. (1999) with tricothecene-producing strains of *Fusarium* sp., as an important tool to differentiate aflatoxin-producing from non-producing strains of *A. flavus* and *A. parasiticus*. Indeed, RT-PCR enables the study of gene expression by allowing the detection of mRNAs transcribed by specific genes owing to PCR amplification of cDNA intermediates synthesized by reverse transcription. The presence or lack of mRNAs could permit direct differentiation between aflatoxinogenic and non-aflatoxinogenic strains. In addition, several specific mRNAs may be detected simultaneously in a single RNA sample by multiplex RT-PCR, with the advantage of having a unique response to the expression of several genes enclosed in the aflatoxin biosynthetic pathway.

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