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PCR Methods for the Detection of Mycotoxin-producing Fungi

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11.1 Introduction

Nearly every food or feed commodity can be contaminated by fungal organisms. It is estimated that approximately 25% of the yearly production of plant-derived foods are spoiled by fungi. Many of the food-borne filamentous fungi are capable of producing mycotoxins, which are toxic metabolites of concern to both the health of humans and the health of animals. Mycotoxins, like antibiotics, are secondary metabolites, which are primarily produced in the idiostage of fungal growth. Some mycotoxins, such as patulin, were originally isolated as antibiotics due to their inhibitory activity towards bacteria, but were later shown to be toxic in animal experiments (Wilson, 1974). Mycotoxins belong to different chemical classes, and mainly polyketide, isoprene and amino acid-containing substances are found (Martin, 1992). Approximately 300 different mycotoxins have been identified, but only about 20 mycotoxins produced by different species are relevant to human health (Table 11.1). Due to the different molecular structures of these mycotoxins, their influences on human and animal health range from teratogenic, immunosuppressive, tremorgenic, nephrotoxic, hepatotoxic to carcinogenic effects (Bullerman, 1979). Some mycotoxicoses are documented by epidemiological studies. Examples are the ergotism due to the ingestion of ergot alkaloides produced by *Claviceps purpurea* (Barger, 1931), the alimentary toxic aleukia which occurred in the Urals and was due to the infection of cereals with T2 toxin-producing fungi (Bamburg *et al.*, 1968), the endemic nephropathia in the Balkan region which correlated with the high occurrence of ochratoxin A-producing filamentous fungi (Krogh, 1987), the high incidence of oesophageal cancer in relation to the high intake

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Table 11.1. Mycotoxins of relevance to human health.

Genus	Toxins
Aspergillus	Aflatoxin Sterigmatocystin Cyclopiazonic acid
Penicillium	Patulin Ochratoxin A Citrinin Penitrem A Cyclopiazonic acid PR toxin
Fusarium	T2 Toxin Deoxynivalenol Nivalenol Zearalenon Diacetoxyscirpenol Fumonisin
Alternaria	Tenuazonic acid Alternariol Alternariol monomethylether
Claviceps	Ergot alkaloids

of fumonisin-containing maize products (Nelson *et al.*, 1993), and some cases of epidemiological correlations between the higher incidence of liver cancer and higher ingestion of aflatoxins (Chu, 1991). Even if these drastic, well-documented cases are rare, the constant uptake of small amounts of mycotoxins, especially those with carcinogenic activity, can have profound effects on human health.

This situation demonstrates the importance of using appropriate methods to control the mycological status of food and feed commodities. Conventional methods for the detection of mycotoxinogenic fungi are culture methods, which are time-consuming and require expertise in fungal taxonomy. Taxonomic classification can be simplified by the use of selective media, but the time required is not reduced (Davies *et al.*, 1987). These problems can be overcome by the use of the PCR which can reduce the detection time from several days to several hours. The diagnostic PCR approach for the detection of mycotoxinogenic fungi is an indirect method. DNA or DNA fragments of the target organism can be amplified and detected by gel electrophoresis. However, the method does not distinguish between dead and living cells. This fact, which is a disadvantage for the detection of pathogenic bacteria in foods, is advantageous in the case of

mycotoxinogenic fungi, as mycotoxins are usually very stable. A positive PCR can, therefore, be taken as an indication that the sample potentially contains mycotoxins and should be analysed further. The PCR approach, however, has the disadvantage that the quantification of fungal biomass is not easy and although some PCR-based quantification methods have been described (Hu *et al.*, 1993), they are too laborious for routine analysis.

A prerequisite for the development of a diagnostic PCR is the availability of unique target sequences, which are specific for the mycotoxin-producing fungus. Ideally, these target sequences should not be present in strains of the same species that are not able to produce mycotoxins. Possible target sequences for PCR or gene probe approaches are given in Table 11.2. For detection of pathogenic or toxinogenic bacteria in foods, respective toxin or virulence genes are often used as target sequences for amplification (Olsen *et al.*, 1996). The counterparts for mycotoxinogenic fungi are the genes which code for the enzymes of the mycotoxin biosynthetic pathway (referred to as mycotoxin biosynthetic genes). However, to date, only some of the genes of mycotoxin biosynthetic pathways have been cloned and sequenced. The best analysed biosynthetic pathways at the genetic level are those from the aflatoxins (Trail *et al.*, 1995a; Yu *et al.*, 1995), the trichothecens (McCormick *et al.*, 1996), patulin (Beck *et al.*, 1990; Wang *et al.*, 1991), PR-toxin (Procter and Hohn, 1993) and for sterigmatocystin (Kelkar *et al.*, 1996). Sterigmatocystin is a precursor of aflatoxin and the sterigmatocystin biosynthetic genes are rather homologous to the aflatoxin biosynthetic genes (Brown *et al.*, 1996). Genes for biosynthetic enzymes of secondary metabolites are usually clustered (Hohn *et al.*, 1993) and this is true for the aflatoxin (Trail *et al.*, 1995a; Yu *et al.*, 1995), sterigmatocystin (Brown *et al.*, 1996), trichothecene (Hohn *et al.*, 1993) and fumonisin biosynthetic genes (Desjardins *et al.*, 1996).

The polyketide synthase gene from *Penicillium patulum*, which is the key enzyme in the biosynthetic pathway of the mycotoxin patulin, has been cloned (Beck *et al.*, 1990). Polyketide synthases are often involved in fungal secondary metabolism and can be found in various species (Hopwood and Sherman, 1990). The polyketide synthases are composed of different catalytic domains, each specific to a certain reaction. These domains, however, often

Table 11.2. Target sequences for PCR/gene probes.

Specificity	Target sequences	Microorganisms
Gene specific	Toxin genes, virulence genes	Bacteria
	Toxin biosynthetic genes	Fungi
Empirical	Ribosomal genes, random cloned fragments, RAPD products	Bacteria and fungi

share high sequence homology, even between species, which makes this gene unsuitable as a specific target gene for diagnostic PCR (Mayorga and Timberlake, 1992).

11.2 Mycotoxin Biosynthetic Genes as Target Sequences

11.2.1 Aflatoxinogenic fungi

The aflatoxins are the most carcinogenic natural substances known and can be found in various food commodities (Diener *et al.*, 1987). They are produced predominately by *Aspergillus flavus*, *A. parasiticus* and *A. nomius* (Bennett, 1988). Whereas nearly all *A. parasiticus* isolates are able to produce aflatoxins B_1, B_2, G_1 and G_2, *A. flavus* is only able to produce aflatoxins B_1 and B_2. Only about 40–50% of *A. flavus* strains isolated are able to produce these mycotoxins. *A. flavus* is highly related taxonomically to *A. oryzae* as is *A. parasiticus* to *A. sojae* (Kurtzman *et al.*, 1986) and they share 90% nucleotide sequence homology. *A. oryzae* and *A. sojae* are used in the food industry as fermentation organisms and both are generally recognized as safe (GRAS) organisms. Both species have never been reported to produce aflatoxin, but they show high morphological similarity to their aflatoxin-producing relatives (Klich *et al.*, 1995). A method specific to aflatoxin-producing fungi should be capable of differentiating between these closely related species and also between aflatoxin-producing and non-producing *A. flavus* strains.

The aflatoxin biosynthetic gene cluster is partially elucidated. The sequences of some of the genes are already known and published (Trail *et al.*, 1995b). Interestingly, the genes are organized in such a way that the gene encoding the first enzyme in the pathway is located at one end of the cluster and the other genes follow in the same order as the enzymatic reactions in the biosynthetic pathway (Fig. 11.1). Due to this arrangement, the gene for norsolorinic acid reductase (*nor-1*) is located at one end of the cluster, the gene for versicolorin A dehydrogenase (*ver-1*) lies in the middle, and the gene for sterigmatocystin-*O*-methyltransferase is located at the other end of the cluster. By selecting these genes as target sequences in a multiplex PCR, the whole aflatoxin biosynthetic gene cluster can be covered, and partial deletions of the cluster can be detected (Geisen, 1996). Conventional morphological methods for the detection of aflatoxinogenic fungi cannot distinguish between aflatoxin producing and non-producing strains. If the non-producing phenotype is due to a deletion of the biosynthetic gene cluster or a part thereof, or to nucleotide changes at the primer binding sites, the PCR approach is able to distinguish between both genetic alterations.

In a multiplex PCR system, several genes can be detected in one reaction at a time. Several primer sets are added to the reaction mixture and the reaction is carried out at an optimized temperature. After electrophoresis of

the products, several amplicons become visible. Multiplex PCR with up to 18 primers has been described (Chamberlain *et al.*, 1989). The advantage of this system is improved specificity. It was observed that some secondary metabolite biosynthetic genes are more widely distributed in different fungal species than expected (see later for details). Thus, it might happen that a PCR product of the same length as the diagnostic PCR product may occur in a mycotoxin non-producing species. This situation reduces specificity drastically if only a monomeric PCR is used. The occurrence of two or more

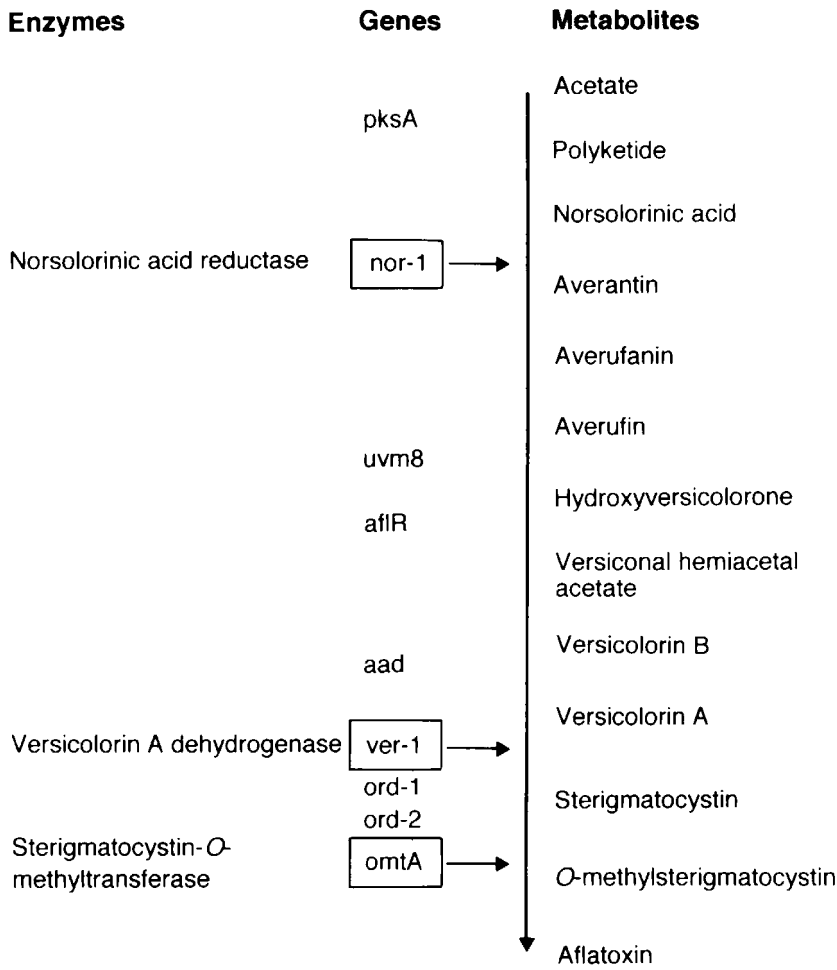

Fig. 11.1. Schematic illustration of the aflatoxin biosynthetic pathway. The order of the genes reflects their relative location in the aflatoxin biosynthetic gene cluster, according to Yu *et al.* (1995). The target genes of the multiplex PCR analysis are indicated in boxes.

PCR products of identical length as the diagnostic PCR products with template DNA of non-producing species, however, is unlikely. In addition, a multiplex reaction has the advantage that an internal control reaction can be included which signals the activity of the polymerase.

Due to the complexity of a multiplex PCR system, several requirements must be met. The reaction kinetics of the different primer sets should be similar to ensure that comparable amounts of PCR products are produced during a reaction. The reaction kinetics are strongly dependent on the primer design: G/C content, melting temperature, secondary structures, 5′3′ overlap. A G/C content of 40–60% and a length of 23–28 nucleotides are suggested as general guidelines for specific annealing. The primary structure of published aflatoxin biosynthetic gene-specific primers are given in Table 11.3.

Another important aspect for the development of a multiplex reaction is the primer location. They should be chosen in such a way that the resulting PCR products are easily separated by agarose gel electrophoresis. The primer binding sites for the detection of aflatoxinogenic fungi with the multiplex PCR system are shown in Fig. 11.2. The presence of introns in two of the amplicons does not influence the result. This indicates that the intron position and length are very conserved in aflatoxin biosynthetic genes. The

Table 11.3. Sequences of published primers specific for aflatoxin biosynthetic genes.

Gene	Sequence[a]	Position[b]	Reference
aflR >	TATCTCCCCCCGGGCATCTCCCGG	450	Shapiro *et al.*
aflR <	CCGTCAGACAGCCACTGGACACGG	1482	(1996)
nor1 >	ACCGCTACGCCGGCACTCTCGGCAC	501	Geisen (1996)
nor1 <	GTTGGCCGCCAGCTTCGACACTCCG	900	
ver1 >	ATGTCGGATAATCACCGTTTAGATGGC	496	Shapiro *et al.*
ver1 <	CGAAAAGCGCCACCATTCACCCCAATG	1391	(1996)
ver1 >	GCCGCAGGCCGCGGAGAAAGTGGT	623	Geisen (1996)
ver1 <	GGGGATATACTCCCGCGACACAGCC	1160	
omtA >	GGCCCGGTTCCTTGGCTCCTAAGC	208	Shapiro *et al.*
omtA <	CGCCCCAGTGAGACCCTTCCTCG	1232	(1996)
omtA >	GTGGACGGACCTAGRCCGACATCAC	301	Geisen (1996)
omtA <	GTCGGCGCCACGCACTGGGTTGGGG	1098	

[a]The sequences are given 5′ to 3′.
[b]The positions of the first and last nucleotide of the amplified product are given relative to the first codon of the gene.

results of typical multiplex PCR analyses with DNA from aflatoxinogenic fungi are shown in Fig. 11.3. All aflatoxin-producing strains show the same triplet pattern after electrophoretic separation of the PCR products. The pattern of the aflatoxinogenic *A. flavus* strains was identical to that of the *A. parasiticus* strains, indicating the homology of the aflatoxin biosynthetic genes in both species, which is also described in the literature (Yu *et al.*, 1995). Non-aflatoxinogenic *A. flavus* strains gave variable results. One strain showed no signal at all, indicating a complete or nearly complete deletion of the aflatoxin biosynthetic gene cluster. Another strain exhibited a doublet pattern, the band for the *omt-A* gene was missing, suggesting a deletion of the part of the gene cluster containing the genes for the last reactions in the biosynthetic pathway. A third strain possessed all three PCR DNA product bands, demonstrating another type of mutation, perhaps in one of the regulatory genes. Most of the non-aflatoxinogenic strains which were analysed showed changes in the triplet pattern, suggesting that their phenotype was due to deletions of the aflatoxin biosynthetic genes.

A. versicolor, which is not an aflatoxin-producing species, showed the same triplet pattern. This was not unexpected as *A. versicolor* is a sterigmatocystin producer, and sterigmatocystin is the precursor of aflatoxin (Dutton, 1988). It is synthesized as an end-product of a secondary metabolite pathway in some fungi like *A. versicolor*, *A. nidulans*, and in species of *Chaetomium*, *Monocillium*, *Farrowia* and *Bipolaris*. Sterigmatocystin by itself is an important mycotoxin, although with a reduced toxicity compared with aflatoxin (Feuell, 1969). The sterigmatocystin biosynthetic pathway is very similar to the one for the aflatoxin biosynthesis. For example, the genes for the conversion of versicolorin A to sterigmatocystin are very conserved in both pathways. In addition, *aflR*, a regulatory gene of the aflatoxin biosynthetic pathway, is able to induce the genes of the sterigmatocystin

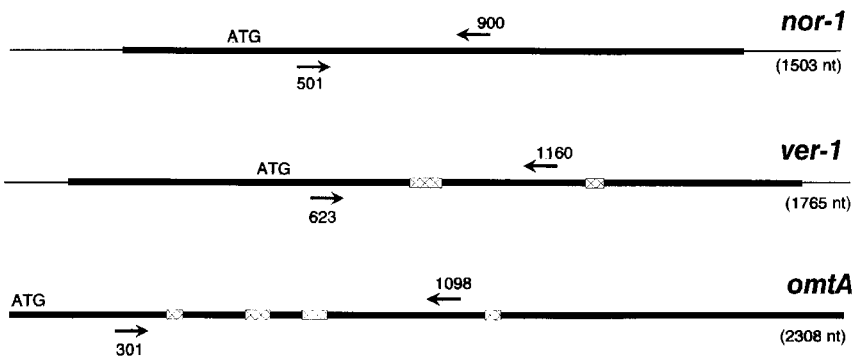

Fig. 11.2. Illustration of the primer binding sites of the three aflatoxin biosynthetic gene-specific primer pairs used for multiplex PCR. Thick lines represent the aflatoxin biosynthetic gene coding sequences, cross-hatched boxes indicate intron sequences.

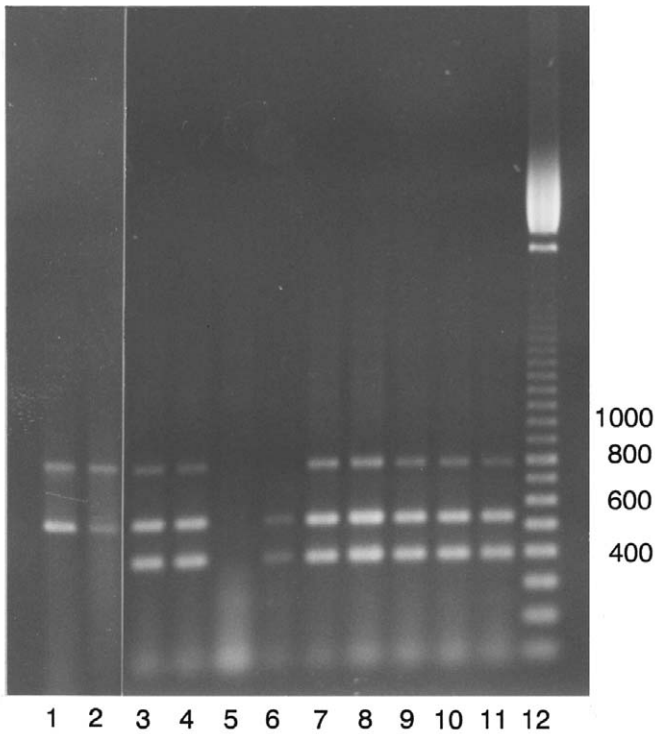

Fig. 11.3. Agarose gel of the multiplex PCR products of different *A. flavus, A. parasiticus, A. versicolor, A. sojae* and *A. oryzea* strains. Chromosomal DNA of these strains was isolated and subjected to PCR analysis using the aflatoxin biosynthetic gene-specific primer. Lane 1, *A. sojae* DSM1147; lane 2, *A. oryzea* DSM1861; lane 3, *A. versicolor* BFE294; lanes 4–6, *A. flavus* BFE301, BFE310, BFE311; lanes 7–9, *A. flavus* BFE84, BFE292, BFE302; lanes 10 and 11, *A. parasiticus* BFE291, BFE293; lane 12, size standard, fragment sizes are indicated in bp.

biosynthetic pathway when introduced into a mutant of *A. nidulans*. A gene for a polyketide synthase which is required for aflatoxin biosynthesis was also identified in *A. nidulans* (Brown *et al.*, 1996).

As mentioned above, the method should differentiate between *A. flavus*, *A. parasiticus* and the related species, *A. oryzae* and *A. sojae*. Figure 11.3 shows that this is, indeed, the case. Both fermentation organisms obviously carry only a part of the aflatoxin biosynthetic gene cluster, as two bands specific for the *ver-1* and *omt-A* genes appear. But the band for the *nor-1* gene is missing, indicating a deletion of the beginning of the gene cluster. The method clearly differentiates between these two GRAS organisms and the aflatoxinogenic fungi. These organisms do not have the same habitat and *A. oryzae* and *A. sojae* are only scarcely found in nature. Both strains are adapted to a fermentative food environment, which is not the typical habitat

for aflatoxinogenic species. They are, however, morphologically very similar to the aflatoxinogenic species and the ability of the PCR method to differentiate between these species can be used for the confirmation and safety control of the GRAS species used for production of human food.

This diagnostic PCR method gave negative results with many of the food-borne fungal species tested (Table 11.4). These included different species of the genera *Penicillium*, *Fusarium*, *Aspergillus*, *Byssochlamys* and *Geotrichum*. Two interesting exceptions were several strains of *Penicillium roqueforti* and *P. italicum*. Both species gave a positive signal in the case of the *nor-1* specific band. The PCR product which appeared had the same migration behaviour as the *nor-1*-specific amplicon from an aflatoxinogenic strain. To analyse potential sequence homology, a digoxigenin-labelled *nor-1* PCR product was used as a probe for hybridization to the PCR product from *P. roqueforti*. A weak signal indicated some homology, suggesting that *P. roqueforti* contains sequences with homology to the *nor-1* gene of aflatoxinogenic fungi. The results demonstrate the advantage of multiplex PCR analyses over monomeric reactions with regard to their specificity.

Shapiro *et al.* (1996) described a similar diagnostic PCR method for the detection of aflatoxinogenic fungi. Three target genes, the *omt-1* gene, the *ver-1* gene, and the *apa-2* gene, from the aflatoxin biosynthetic gene cluster were used in three separate monomeric PCRs. The *apa-2* (now named as *aflR*) gene is a regulatory gene, influencing the expression of the other

Table 11.4. Results of PCR analyses specific for aflatoxin biosynthetic genes with food-borne fungi.

Species	BFE strain[a]	*nor-1*	*ver-1*	*omt-A*
Penicillium italicum	BFE45	+	–	–
P. digitatum	BFE46	–	–	–
P. nalgiovense	BFE66	–	–	–
P. camemberti	BFE135	–	–	–
P. chrysogenum	BFE236	–	–	–
P. roqueforti	BFE278	+	+[c]	–
Byssochlamys nivea	BFE218	–	–	–
Geotrichum candidum	BFE222	–	–	–
Fusarium moniliforme	BFE322	–	–	–
F. graminearum	DSM1095[b]	–	–	–
F. poae	DSM62376	–	–	–
F. solani	DSM62416	–	–	–
F. sporotrichoides	DSM62423	–	–	–

[a]Strain number according the culture collection of the Bundesforschungsanstalt für Ernährung.
[b]Strain number of the German Culture Collection (DSM).
[c]The PCR fragment was slightly larger than that of *A. parasiticus*.

aflatoxin biosynthetic genes (Chang *et al.*, 1993). In contrast to the results described by Geisen (1996), no strong positive PCR signals were found with DNA from aflatoxinogenic *A. flavus* strains. After 30 cycles a weak signal for the *omt-1* gene appeared, and after 40 cycles, a weak band for the *ver-1* gene became visible with DNA from *A. flavus* as the template, but a reaction with the *aflR*-specific primer pair failed. The sequences for the *aflR* genes of *A. flavus* and *A. parasiticus* are highly homologous, but show distinct differences according to Chang *et al.* (1995). These sequence dissimilarities might be responsible for the failure to detect the *aflR* gene in *A. flavus* by PCR. Shapiro *et al.* (1996) could not identify the *omt-1* gene in *A. nidulans*, a potential sterigmatocystin-producing species, and they argued that no *omt-1* gene was present. The PCR approach described by Geisen (1996) however, demonstrates that at least in *A. versicolor*, an *omt-1*-homologous sequence was present.

Detection of aflatoxinogenic A. flavus *strains in a food system*

Aflatoxinogenic fungi are found predominantly in certain foods such as peanuts, almonds, figs and spices (Bullermann, 1979). Figs are often infected by members of the group of black aspergilli, including *A. flavus* and *A. parasiticus* (Doster *et al.*, 1996). Figs that were infected with an aflatoxinogenic strain of *A. flavus* could be identified with the PCR approach. The expected amplicons were generated with DNA isolated from infected figs, whereas uninfected figs gave no signal at all (Färber *et al.*, 1997).

The most crucial prerequisite for diagnostic PCR with food samples is the isolation of sample DNA in a PCR-usable form. It is known that certain food components like fats and proteins can interfere with the activity of *Taq* polymerase (Rossen *et al.*, 1992). An inhibition of the PCR was also observed for the infected figs, indicating that a high concentration of carbohydrates is also inhibitory to the PCR system. The sample DNA was prepared by a phenol extraction method. An estimation of the sensitivity of the reaction revealed that it was reduced by a factor of ten compared with pure fungal DNA.

Kreutzinger *et al.* (1996) described a method for reducing inhibitory activity in PCR from environmental samples. In an attempt to detect ectomycorrhizal fungi in their natural habitat by PCR, they observed that the *Taq* polymerase was inhibited strongly by compounds in the sample. This problem was overcome by the use of very dilute template DNA in a first round of PCR, followed by a second reaction with nested PCR primers. Lantz *et al.* (1994) have described another approach for the preparation of sample DNA. They attempted to detect pathogenic *Listeria monocytogenes* cells in soft cheese by PCR. However, with conventional sample DNA preparation protocols, the reaction failed; foods with a high fat content are particularly inhibitory to the polymerase reaction. Functional template DNA can be prepared if an aqueous, two phase sample preparation system is used. With this system, the sample is homogenized in a mixture of two

non-soluble aqueous solutions, which separate in two phases after homogenization. One phase consists of a polyethylene glycol (PEG) solution and the other of a dextran solution. This extraction system separates PCR inhibitory compounds, which partition to the PEG phase. If samples from the dextran phase are used for PCR, *L. monocytogenes* cells can be detected.

Shapiro *et al.* (1996) used their PCR method for the detection of aflatoxinogenic fungi in corn. They described an enrichment procedure to amplify the template DNA prior to PCR by suspending the corn sample in a rich medium; the sensitivity of the method increased with the incubation time but the detection time increased simultaneously. Only corn inoculated with aflatoxinogenic *A. parasiticus* gave positive signals with the PCR and after 24 h incubation, 1×10^2 spores of *A. parasiticus* could be detected.

Even with sophisticated DNA preparation protocols, the occurrence of false negative reactions with food samples remains a problem. The use of an internal standard can give information about the quality of the reaction. If a universal primer set is used which gives a PCR product with all food-relevant fungal species, then a DNA band of a particular length should occur in nearly all food samples where there is contaminating fungal biomass. Alternatively, the sample can be spiked with purified fungal DNA of a certain species. The occurrence of the standard amplicon is then an indication of the efficiency of the reaction. It is advantageous to use a universal primer set instead of a specific one, as every available fungal DNA can then be used as an internal standard. A universal fungus-specific primer system has been described by Kappe *et al.* (1996); the target sequences of this system are the 17S ribosomal RNA genes. Similarly, the conserved nucleotide sequences of the rRNA genes, which can serve as primer sequences for the amplification of the ITS1 and ITS2 regions, have been described (White *et al.* 1990).

The primers ITS1 and ITS2 can be added as a fourth primer pair to the multiplex PCR. They amplify the ITS1 region from different fungal species and the PCR product is approximately 250 bp long, although the length may vary depending on the template DNA used. Figure 11.4 shows some typical results. The aflatoxinogenic strain now shows a quadruplet pattern as expected. All other strains, including different *Penicillium* or *Aspergillus* species, show only the standard band of the ITS1 region. As a result of this reaction design, a sample with a quadruplet pattern indicates the presence of an aflatoxinogenic species, whereas a monomeric band of about 250 bp indicates that the reaction was not inhibited by food components and that the sample did not contain potential aflatoxinogenic fungi.

11.2.2 PR toxin producing fungi

Penicillum roqueforti occurs in various natural habitats. It is used as a fungal starter culture for the production of blue-veined cheese (Marth, 1987), but can also be found as a spoilage organism in different food and feed commodities (Frisvad, 1988). *P. roqueforti* is a heterogeneous species that

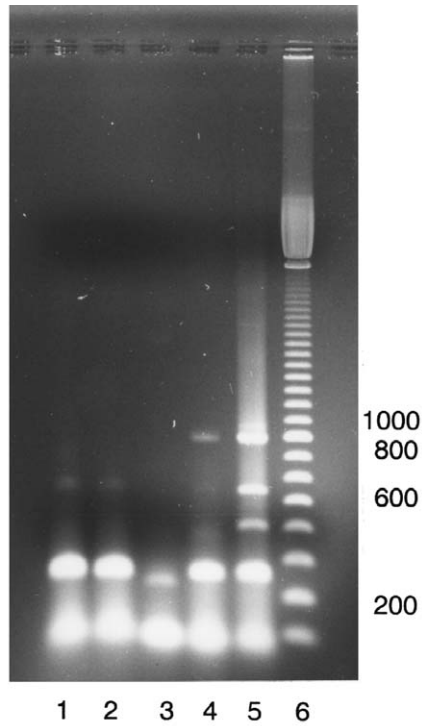

Fig. 11.4. Agarose gel of the multiplex PCR products of different food-borne fungi. Chromosomal DNA of these strains was isolated and subjected to PCR analysis using the aflatoxin biosynthetic gene-specific primers in addition to a primer pair specific for the ITS1 region of the rRNA genes. Lane 1, *F. moniliforme* BFE312; lane 2, *F. solani* BFE325; lane 3, *P. digitatum* BFE350; lane 4, *A. flavus* BFE312 (aflatoxin negative); lane 5, *A. flavus* BFE84 (aflatoxin positive); lane 6, size standard, fragment sizes are indicated in bp.

was recently divided, according to secondary metabolite patterns and differences in its ITS regions, into three distinct species: *P. roqueforti*, *P. paneum* and *P. carneum* (Boysen *et al.*, 1996). All three species are able to produce various secondary metabolites: *P. carneum* is capable of producing patulin, penitrem A and mycophenolic acid; *P. roqueforti* produces PR toxin, marcfortines and fumigaclavine A; and *P. paneum* produces patulin, botryodiploidin and other secondary metabolites. All three species are morphologically very similar. *P. roqueforti* is the species which is used exclusively for the production of blue cheese. All strains isolated from blue cheese are able to produce PR toxin (Geisen and Holzapfel, 1995). PR toxin is a relatively toxic substance, which is able to inhibit protein translation in eukaryotic cells (Moule *et al.*, 1978). However, PR toxin is not stable in the cheese matrix where it is converted to the much less toxic PR imine (Scott, 1981). Nevertheless, strains which do not produce PR toxin are preferable over

conventional strains with respect to safety considerations. PR toxin is a sesquiterpenoid secondary metabolite, with acetyl-CoA as the first precursor. One of the key enzymes in its biosynthetic pathway is the aristolochen synthase, a sesquiterpene cyclase. The nucleotide sequence of this gene is known (Procter and Hohn, 1993) and, by using gene-specific PCR with the aristolochen synthase gene (*ari1*) as a target, it is possible to screen *P. roqueforti* strains for the presence of aristolochen synthase. Strains with a deletion in that gene are expected to be unable to produce PR toxin. This method of screening for PR toxin-free strains is more direct, reliable and less time-consuming than looking for the ability to produce PR toxin especially as PR toxin production is dependent on various environmental conditions (Scott *et al.*, 1977), which makes the identification of PR toxin-negative strains by conventional methods difficult.

Strains isolated from cheese and infected feeds were subjected to PCR analysis with *ari1*-specific primers. A product of the expected length occurred in nearly all strains, indicating the presence of the *ari1* gene. Figure 11.5 shows a typical result obtained with several strains. Subsequent analysis by thin layer chromatography (TLC) showed that all strains except one produced PR toxin. The strains which were negative in the PCR, but which were able to produce PR toxin, were verified by dot-blot hybridization using an *ari1*-specific probe. After the analysis all PR toxin-producing strains were positive either in the dot-blot hybridization or the PCR analysis (Table 11.5). An exception was one strain which did not produce PR toxin, indicating that it did not carry the *ari1* gene. The other strains which were negative in the PCR analysis, but positive in the dot-blot hybridization and TLC obviously carried active genes which may have alterations in their primer binding sites. These results show that it is not always appropriate to rely solely on one PCR target site, as small changes in the sequence can have dramatic effects on the PCR results. The single strain which was negative in the PCR analysis and did not produce PR toxin, was originally isolated from cheese and is therefore probably a safe candidate for the production of blue-veined cheese.

As mentioned above, homologues of aflatoxin biosynthetic genes can be identified unexpectedly in *P. roqueforti* (Table 11.5). The same phenomenon is apparent with the *ari1* gene and a sequence homologous to that gene can be found unexpectedly in various species (Table 11.6). The gene-specific PCR gave positive results with *P. camemberti* and *Byssochlamys nivea*, both species which are not known to produce PR toxin. With the dot-blot analysis even more species gave positive results, indicating the occurrence of *ari1*-like sequences in a variety of species, which are not able to produce PR toxin. The relevance of the frequent occurrence of a nucleotide sequence homologous to secondary metabolite biosynthetic genes in various fungi is not known. Enzymes of secondary metabolite pathways have less specificity than their counterparts from primary metabolism (Dutton, 1988) and this results in a somewhat different behaviour from that of biosynthetic enzymes of primary metabolism. In certain cases, one enzyme can catalyse analogous reactions

with related but different substrates. This can result in a metabolic grid, a network of enzymatic reactions from different substrates that give rise to different reaction products (Yabe *et al.*, 1991). This is one of the reasons why in secondary metabolism a large number of end-products can be produced, such as the aflatoxins, the fumonisins, the trichothecens or the ochratoxins. This behaviour may also be an explanation for the positive PCR results for specific secondary metabolite biosynthetic genes in various species. If these species possess active genes with homology to mycotoxin biosynthetic genes,

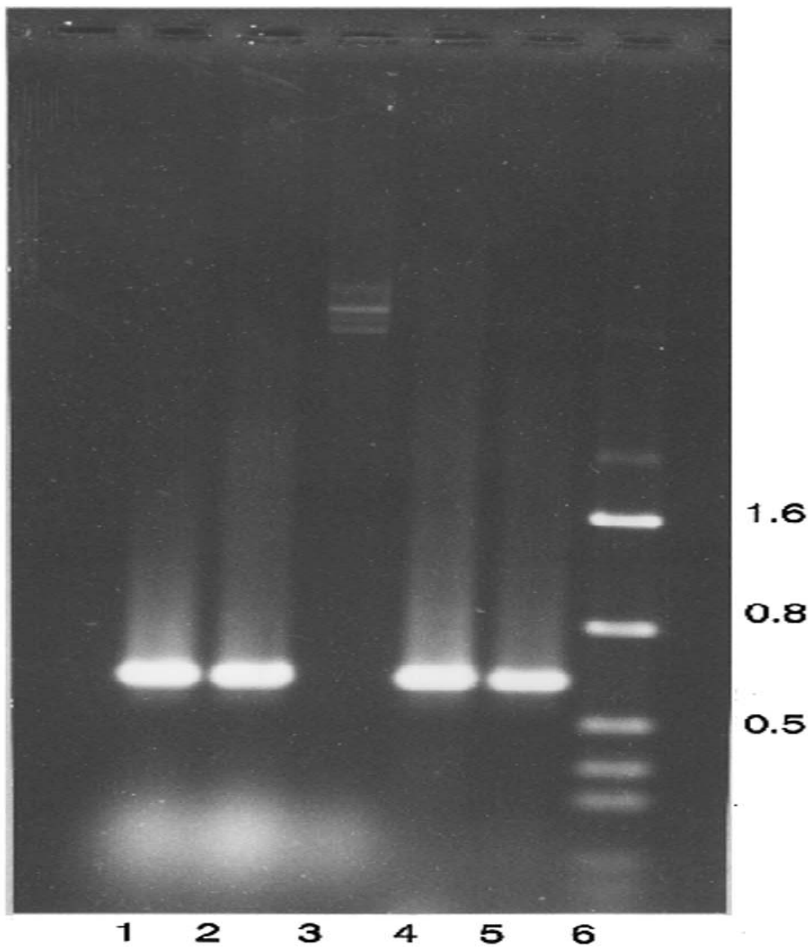

Fig. 11.5. Agarose gel of PCR products of *P. roqueforti* strains and a *P. nalgiovense* strain (as a control) with aristolochen synthase-specific primers. Lane 1, *P. roqueforti* BFE42; lane 2, *P. roqueforti* BFE50; lane 3, *P. nalgiovense* BFE66; lane 4, *P. roqueforti* BFE53; lane 5, *P. roqueforti* BFE54; lane 6, size standard, fragment sizes are indicated in kb.

Table 11.5. Presence of the *ari1* gene sequences and ability to produce PR toxin in various *Penicillium roqueforti* strains.

BFE strain[a]	*ari1*[b]	Dot-blot[c]	PR toxin[d]	*nor1*[e]	Source[f]
BFE42	+	+	+	–	C
BFE53	+	+	+	+	C
BFE168	+	+	+	ND	C
BFE169	+	+	+	–	C
BFE170	+	+	+	ND	C
BFE171	+	+	+	ND	C
BFE172	+	+	+	ND	C
BFE50	–	+	+	–	C
BFE54	–	–	–	–	C
BFE62	–	+	+	+	C
BFE63	+	+	+	+	C
BFE208	+	+	+	ND	S
BFE209	+	+	+	ND	S
BFE210	+	+	+	ND	S
BFE211	+	+	+	ND	S
BFE215	+	+	+	ND	S
BFE216	+	+	+	–	S
BFE212	–	+	+	–	S
BFE213	+	+	+	–	S
BFE284	+	+	+	+	S
BFE285	+	+	+	+	S

[a]Strain number according the culture collection of the Bundesforschungsanstalt für Ernährung.
[b]The presence of the *ari1* gene sequences was checked with gene-specific PCR.
[c]For dot-blot analysis the labelled *ari1*-specific PCR product was used.
[d]Detected by thin layer chromatography.
[e]The presence of the *nor1* gene was checked by gene-specific PCR.
[f]C, isolated from cheese; S, isolated from silage.
ND, not determined.

their gene products may participate in secondary metabolite pathways leading to different, perhaps as yet unidentified, end-products.

These results taken together suggest that a monomeric PCR reaction, which targets only one gene, possesses insufficient specificity for the detection of mycotoxin-producing fungi. Multiplex reactions, as was described for aflatoxinogenic fungi, are recommended. If only one gene of the mycotoxin biosynthetic pathway is known, as in the case of PR toxin, another known specific sequence can be used as an additional target sequence, for example the sequences of the rRNA genes of *P. roqueforti* (Boysen *et al*., 1996).

Table 11.6. Presence of aristolochen synthase gene like nucleotide sequences in various species.

	Detection of *ari1*-like sequences by	
BFE strain[a]	PCR	Dot-blot
P. roqueforti BFE278	+	+
P. camemberti BFE135	+	+
B. nivea BFE218	+	+
P. italicum BFE45	–	+
P. nalgiovense BFE66	–	+
A. nidulans BFE125	–	+
P. digitatum BFE46	–	–
P. chrysogenum BFE236	–	–
G. candidum BFE222	–	–
A. flavus BFE388	–	–
A. parasiticus BFE293	–	–
A. versicolor BFE294	–	–

[a]Strain number according the culture collection of the Bundesforschungsanstalt für Ernährung.

11.2.3 rDNA and miscellaneous sequences as target sequences

The organization of ribosomal RNA genes is highly conserved within the fungal kingdom. The small subunit, 5.8S, and large subunit rRNA genes are organized in a single transcription unit and are separated by two spacer regions ITS1 and ITS2. The RNA transcript is post-transcriptionally processed into small subunit, 5.8S, and large subunit rRNA species. The rRNA sequences have conserved and variable regions and are useful for studying distantly related organisms at the taxonomic level. The ITS sequences, however, are more variable in their nucleotide composition and can be used for differentiation of species or populations. It may be possible to identify unique sequences within the ITS regions which may serve as target sequences for diagnostic PCR. Universal primers which can be used for amplification or cycle sequencing of ITS1 and ITS2 have been described (White *et al.*, 1990; Fig. 11.6). It is relatively straightforward to sequence these regions by using primers such as ITS1 and ITS4, and to determine unique sequences for primer development. A prerequisite for usage of these sequences for detection of mycotoxinogenic fungi is the requirement that their presence be linked to the ability of a strain to produce a mycotoxin. This is one of the drawbacks of this approach as in most cases not all strains of a species are able to produce the respective mycotoxin. A species-specific primer pair identified by this approach does not ultimately mean that it is

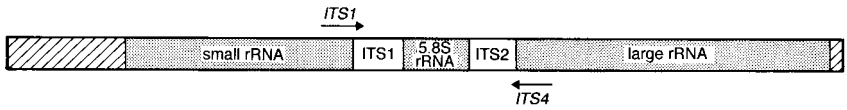

Fig. 11.6. Illustration of a typical fungal rRNA gene and the position of the universal primers ITS1 and ITS4.

specific only for the mycotoxinogenic strains of that species. A positive result from PCR of the rRNA genes is, therefore, not as valid as the results from a PCR targeted to the mycotoxin biosynthetic genes.

Considerable literature data on ITS sequences of different fungi are available. Most of the sequences were obtained for taxonomic purposes and their specificity in PCR analyses has not been determined (Morales *et al.*, 1993; Bunting, *et al.*, 1996; Waalwijk *et al.*, 1996). *F. sambucinum* (teleomorph, *Gibberella pulicaris*) is able to produce fusarin C and trichothecenes (Smith and Solomons, 1994). O'Donnel (1997) has described three different groups of ITS sequences within the species *F. sambucinum*. However, the possible relationship between these ITS groups and the ability to produce a mycotoxin was not considered. Grimm and Geisen (1996) sequenced the ITS1 region of fumonisin-producing *Fusarium* species. They were able to identify regions which were common to all fumonisin-producing fungi, but which showed minor polymorphisms in sequence compared with non-fumonisin-producing species. On the basis of these differences, primer pairs were derived which gave only the expected products from *F. moniliforme*, *F. nygamai*, *F. napiforme* and *F. proliferatum*, i.e. potential fumonisin-producing fungi.

Schilling *et al.* (1996) analysed the ITS region of *Fusarium culmorum* (teleomorph, unknown), *F. graminearum* (teleomorph, *Gibberella zeae*) and *F. avenaceum* (teleomorph, *G. avenacea*) with the aim of developing ITS-specific PCR primers. All three species are plant pathogens of cereals and grasses. The three species are able to produce different mycotoxins: *F. avenaceum* produces moniliformin and trichothecene A; *F. graminearum* produces zearalenone, trichothecene A + B and fusarin C; and *F. culmorum* produces zearalenone, trichothecene A + B and culmorin. Schilling *et al.* (1996) were not able to identify sufficient sequence polymorphisms within the ITS region of these three species to identify specific primer target sites. For this reason, they applied another approach in which preselected random sequences were chosen as target sequences for the PCR primers. This preselection was achieved by identifying RAPD–PCR patterns which were specific for each species. RAPD–PCR is a variation of conventional PCR (Williams, 1990) in which one primer of arbitrary sequence is used. The hybridization temperature during RAPD–PCR is considerably reduced compared with conventional PCR. This enables semi-specific binding of the

primer and results in a specific RAPD pattern. If identical conditions are used, the same pattern should be generated with each reaction. Depending on the type of random primer used, the resulting RAPD pattern can be species-specific (Guthrie *et al.*, 1992), and can differentiate between various genotypes within a species (Hamelin *et al.*, 1993) or even between strains of the same species (Bidochka *et al.*, 1994). The probability that certain bands of the RAPD pattern which occur only in mycotoxin-producing fungi consists of unique sequences is very high. Schilling *et al.* (1996) isolated unique bands of the RAPD pattern of *Fusarium* species. They derived specific PCR primer target sites from their sequences which could be used for differentiation and detection of these species.

The approach described above may be a general method for the development of a diagnostic PCR method for a particular group of microorganisms if no genetic data are available. The same approach was used for the development of gene probes for potential fumonisin-producing fungi (Geisen, 1997). Two RAPD bands were identified which only occurred with potential fumonisin-producing fungi, such as *F. moniliforme*, *F. nygamai*, *F. napiforme* and *F. proliferatum*. These two DNA fragments were labelled either with digoxigenin or with biotin and used as probes for various fungal DNA sequences. Specific hybridization of the probes was visualized by alkaline phosphatase reactions with either fast blue B (resulting in a blue colour) or fast red TR (resulting in a red colour). When both colours were present on the membrane the result was interpreted as the presence of a potential fumonisin-producing fungus. A survey of the results with the DNA from various food-borne fungi is given in Table 11.7. From these results it can be concluded that primer sequences for diagnostic PCR can be derived from RAPD fragments as also shown by Schilling *et al.* (1996).

In addition to rRNA genes or random sequences, sequences from cloned genes of mycotoxin-producing fungi may serve as targets for diagnostic PCR. Niessen and Vogel (1997) developed a PCR analysis which was specific for *F. graminearum*. All other *Fusarium* species or species of other genera gave negative results, indicating high specificity for the method. The reaction was directed against the endogenous galactose oxidase gene (*gaoA*); however, the authors did not attempt to correlate mycotoxin production and PCR results.

11.3 Conclusions

PCR is a valuable tool for the rapid screening of foods and feeds for the presence of mycotoxin-producing fungi. Results can be obtained from an infected sample within 24 h as compared with up to several days by conventional methods. If an appropriate target sequence is chosen, the method can also differentiate between mycotoxin-producing and non-producing strains of a species in most cases.

Table 11.7. Dot-blot analysis with the RAPD gene probes for fumonisin-producing fungi.

BFE strain[a]	Probe 1	Probe 2
Penicillium italicum BFE45	–	–
P. digitatum BFE46	–	–
P. nalgiovense BFE66	–	–
Aspergillus flavus BFE84	–	+
A. nidulans BFE125	–	–
P. camemberti BFE125	–	–
Byssochlamys nivea BFE218	–	–
Fusarium moniliforme BFE314	+	+
F. moniliforme BFE315	+	+
F. moniliforme BFE316	+	+
F. moniliforme BFE317	+	+
F. moniliforme BFE318	+	+
F. moniliforme BFE319	ND	+
F. moniliforme BFE320	+	+
F. proliferatum BFE343	+	+
F. proliferatum BFE344	+	+
F. nygamai BFE353	+	+
F. nygamai BFE355	+	+
F. napiforme BFE354	+	+
F. graminearum BFE323	–	–
F. poae BFE324	–	–
F. solani BFE325	–	–
F. sporotrichoides BFE326	–	–

[a]Strain number according the culture collection of the Bundesforschungsanstalt für Ernährung.
ND, not determined.

Problems with inhibitory substances within food samples can be overcome by an adapted DNA purification protocol (Lantz *et al.*, 1994), the use of highly diluted DNA and a subsequent nested second amplification (Kreutzinger *et al.*, 1996), or controlled by the use of internal standard DNA giving information about the quality of the reaction.

The most direct procedure for the development of a diagnostic PCR method for mycotoxin-producing fungi is the targeting of the mycotoxin biosynthetic genes. If their sequences are not available, other opportunities for the identification of target sequences exist, such as the determination of specificities in ITS sequences, or random sequences preselected by RAPDs; sequences from structural genes may also serve as target sites. These secondary target sequences are less specific because mycotoxin-producing and non-producing strains are indistinguishable. Diagnostic PCR methods

are rapid tools for safety control of foods and feeds. They can, however, only give information about the presence of a potential mycotoxin-producing fungus in a sample but cannot detect the mycotoxin itself. A positive result indicates a potential health hazard and the product should be further checked for the presence of the particular mycotoxin by analytical methods.

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References

Bamburg, J.R., Riggs, N.V. and Strong, F.M. (1968) The structures of toxins from two strains of *Fusarium trincinctum*. *Tetrahedron* 24, 3329–3336.

Barger, G. (1931) *Ergot and Ergotism*. Gurney and Jackson, London.

Beck, J., Ripka, S., Siegner, A., Schiltz, E. and Schweizer, E. (1990) The multifunctional 6-methylsalicylic acid synthase gene of *Penicillium patulum*. *European Journal of Biochemistry* 192, 487–498.

Bennett, J.W. and Papa, K.E. (1988) The aflatoxigenic *Aspergillus* spp. *Advances in Plant Pathology* 6, 263–280.

Bidochka, M.J., McDonald, M.A., St Leger, R.J. and Roberts, D.W. (1994) Differention of species and strains of entomopathogenic fungi by random amplification of polymorphic DNA (RAPD). *Current Genetics* 25, 107–113.

Boysen, M., Skouboe, P., Frisvad, J. and Rossen, L. (1996) Reclassification of the *Penicillium roqueforti* group into three species on the basis of molecular genetic and biochemical profiles. *Microbiology* 142, 541–549.

Brown, D.W., Yu, J., Kelkar, H.S., Fernandes, M., Nesbit, T.C., Keller, N.P., Adams, T.H. and Leonhard, T.J. (1996) Twenty five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. *Proceedings of the National Academy of Sciences USA* 93, 1418–1422.

Bullerman, L.B. (1979) Significance of mycotoxins to food safety and human health. *Journal of Food Protection* 42, 65–86.

Bunting, T.E., Plunbley, K.A., Clarke, B.B. and Hillman, B.I. (1996) Identification of *Magnaporthe poae* by PCR and examination of its relationship to other fungi by analysis of their nuclear rDNA ITS1 region. *Phytopathology* 86, 398–404.

Chamberlain, J.S., Gibbs, R.A., Ranier, J.E., Nguyen, P.N. and Caskey, C.T. (1990) Multiplex PCR for the diagnosis of Duchenne muscular dystrophy. In: Gelfland, D.H., Innis, M.A., Sninsky, J.J. and White, T.J. (eds) *PCR protocols: a Guide to Methods and Applications*. Academic Press, San Diego, pp. 272–281.

Chang, P.K., Cary, J.W., Bhatnagar, D., Cleveland, T., Bennett, J.W., Linz, J., Woloshuk, C.P. and Payne, G.A. (1993) Cloning of the *Aspergillus parasiticus apa-2* gene associated with the regulation of aflatoxin biosynthesis. *Applied and Environmental Microbiology* 59, 3273–3279.

Chang, P.K., Bhatnagar, D., Cleveland, T. and Bennett, J.W. (1995) Sequence variability in homologs of the aflatoxin pathway gene *aflR* distinguishes species in *Aspergillus* section *Flavi*. *Applied and Environmental Microbiology* 61, 40–43.

Chu, F.S. (1991) Mycotoxins: food contamination, mechanism, carcinogenic potential and preventive measures. *Mutation Research* 259, 291–306.

Davies, N.D., Iyer, S.K. and Diener, U.L. (1987) Improved method of screening for aflatoxin with a coconut agar medium. *Applied and Environmental Microbiology* 53,1593–1595.

Desjardins, A.E., Hohn, T.M. and McCormick, S.P. (1993) Trichothecene biosynthesis in *Fusarium* species: chemistry, genetics and significance. *Microbiological Reviews* 57, 595–604.

Diener, U.L., Cole, R.J., Sanders, T.H., Payne, G.A., Lee, L.S. and Klich, M. (1987) Epidemiology of aflatoxin formation by *Aspergillus flavus*. *Annual Review of Phytopathology* 25, 249–270.

Doster, M.A., Michailides, T.J. and Morgan, D.P. (1996) *Aspergillus* species and mycotoxins in figs from California orchards. *Plant Disease* 80, 484–489.

Dutton, M.F. (1988) Enzymes and aflatoxin biosynthesis. *Microbiological Reviews* 52, 274–295.

Färber, P., Geisen, R. and Holzapfel, W.H. (1997) Detection of aflatoxinogenic fungi in figs by a PCR reaction. *International Journal of Food Microbiology* 36, 215–220.

Feuell, A.J. (1969) Types of mycotoxins in food and feeds. In: Goldblatt, L.A. (ed.) *Aflatoxin*. Academic Press, New York, pp. 187–221.

Frisvad, J.C. (1988) Fungal species and their specific production of mycotoxins. In: Samson, R.A. and van Reenen-Hoekstra, E.S. (eds) *Introduction to Food Borne Fungi*. Centraalbureau voor Schimmelcultures, Baarn, pp. 239–249.

Geisen, R. (1996) A multiplex PCR reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. *Systematic and Applied Microbiology* 19, 388–392.

Geisen, R. (1997) Nachweis von potentiell fumonisinbildenden Fusarien mit Hilfe einer 'Multicolour Dot Blot Reaktion'. In: Gareis, M. and Scheuer, R. (eds) *Proceedings of the 18th Mykotoxin Workshop, Bundesanstalt für Fleischforschung*, Kulmbach, 10–12 June 1996. Bundesanstalt für Fleischforschung, pp. 15–20.

Geisen, R. and Holzapfel, W.H. (1995) Molecular charcterization of PR toxin producing *Penicillium roqueforti* strains. *BIOspectrum* 3, 101.

Grimm, C. and Geisen, R. (1996) A PCR reaction specific for potential fumonisin producing *Fusaria* based on ITS sequences. *VAAM Workshop, Regulation in Fungi*. Jülich. November 21–22.

Guthrie, P.A.I., Magill, C.W., Frederiksen, R.A. and Odvody, G.N. (1992) Random amplified polymorphic DNA markers: a system for identifying and differentiating isolates of *Colletotrichum graminicola*. *Phytopathology* 82, 832–835.

Hamelin, R.C., Ouellete, G.B. and Bernier, L. (1993) Identification of *Gremmeniella abietina* races with random amplified polymorphic DNA markers. *Applied and Environmental Microbiology* 59, 1752–1755.

Hohn, T.M., McCormick, S.P. and Desjardins, A.E. (1993) Evidence for a gene cluster involving trichothecene-pathway biosynthetic genes in *Fusarium sporotrichoides*. *Current Genetics* 24, 291–295.

Hopwood, D.A. and Sherman, D.H. (1990) Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. *Annual Review of Genetics* 24, 37–66.

Hu, X., Nazar, R.N. and Robb, J. (1993) Quantification of *Verticillium* biomass in wilt disease development. *Molecular Plant Pathology* 42, 23–36.

Kappe, R., Fauser, C., Okeke, C.N. and Maiwald, M. (1996) Universal fungus-specific primer systems and group specific hybridization oligonucleotides for 18S rRNA. *Mycoses* 39, 25–30.

Kelkar, H.S., Keller, N.P. and Adams, T.H. (1996) *Aspergillus nidulans* stc P encodes an *O*-methyltransferase that is required for sterigmatocystin biosynthesis. *Applied and Environmental Microbiology* 62, 4296–4298.

Klich, M., Yu, J., Chang, P.K., Mullaney, E.J., Bhatnagar, D. and Cleveland, T. (1995) Hybridization of genes involved in aflatoxin biosynthesis to DNA of aflatoxinogenic and non-aflatoxinogenic aspergilli. *Applied Microbiology and Biotechnology* 44, 439–443.

Kreutzinger, N., Podeu, R., Gruber, F., Göbl, F. and Kubiceck, C.P. (1996) Identification of some ectomycorrhizal basidiomycetes by PCR amplification of their *gpd* (glyceraldehyde-3-phosphate dehydrogenase) genes. *Applied and Environmental Microbiology* 62, 3432–3438.

Krogh, P. (1987) Ochratoxins in food. In: Krogh, P. (ed.) *Mycotoxins in Food*. Academic Press, London, pp. 97–121.

Kurtzman, C.P., Smiley, M.J., Robnett, C.J. and Wicklow, D.T. (1986) DNA relatedness among wild and domesticated species in the *Aspergillus flavus* group. *Mycologia* 78, 955–959.

Lantz, P.G., Tjernfeld, F., Borch, E., Hahn-Hägerdahl, B. and Rådström, P. (1994) Enhanced sensitivity in PCR detection of *Listeria monocytogenes* in soft cheese through use of an aqueous two-phase system as a sample preparation method. *Applied and Environmental Microbiology*, 60, 3416–3418.

Marth, E. (1987) Dairy products. In: Beuchat, L. (ed.) *Food and Beverage Mycology*. Van Nostrand Reinhold, New York, pp. 175–209.

Martin, J.F. (1992) Secondary metabolites. In: Kinghorn, J.R. and Turner, G. (eds) *Applied Molecular Genetics of Filamentous Fungi*. Blackie Academic and Professional, London, pp. 214–252.

Mayorga, M.E. and Timberlake, W.E. (1992) The developmentally regulated *Aspergillus nidulans wA* gene encodes a polypeptide homologous to polyketide and fatty acid synthases. *Molecular and General Genetics* 235, 205–212.

McCormick, S.P., Hohn, T.M. and Desjardins, A.E. (1996) Isolation and characterization of *Tri3*, a gene encoding 15-*O*-acetyltransferase from *Fusarium sporotrichoides*. *Applied and Environmental Microbiology* 62, 353–359.

Morales, V.M., Pelcher, L.E. and Taylor, J.L. (1993) Comparison of the 5.8S rDNA and internal transcribed spacer sequences of isolates of *Leptosphaeria maculans* from different pathogenicity groups. *Current Genetics* 23, 490–495.

Moule, Y., Jemmali, M. and Darracq, N. (1978) Inhibition of protein synthesis by PR toxin, a mycotoxin from *Penicillium roqueforti*. *FEBS Letters* 8, 341–344.

Nelson, P.E., Desjardins, A.E. and Plattner, R.D. (1993) Fumonisins, mycotoxins produced by *Fusarium* species: biology, chemistry and significance. *Annual Reviews of Phytopathology* 31, 233–252.

Niessen, L. and Vogel, R.F. (1997) Specific identification of *Fusarium graminearum* by PCR with *gaoA* targeted primers. *Systematic and Applied Microbiology* 20, 111–113.

O'Donnel, K.L. (1992) Ribosomal DNA internal transcribed spacers are highly

divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*). *Current Genetics* 22, 213–220.

Olsen, J.E., Aabo, S., Hill, W., Notermans, S., Wernars, K., Granum, P.E., Popovic, T., Rasmussen, H.N. and Olsvik, Ø. (1995) Probes and polymerase chain reaction for detection of food-borne bacterial pathogens. *International Journal of Food Microbiology* 28, 1–78.

Proctor, R.H. and Hohn, T.M. (1993) Aristolochen synthase: isolation, characterization, and bacterial expression of a sesquiterpenoid biosynthetic gene (*ARi1*) from *Penicillium roqueforti*. *Journal of Biological Chemistry* 268, 4543–4548.

Rossen, L., Nørskov, P., Holmstrøm, K. and Rasmussen, O.F. (1992) Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. *International Journal of Food Microbiology* 17, 37–45.

Schilling, A.G., Möller, E.M. and Geiger, H.H. (1996) Polymerase chain reaction-based assays for species-specific detection of *Fusarium culmorum*, *F. graminearum* and *F. avenaceum*. *Phytopathology* 86, 515–522.

Scott, P.M. (1981) Toxins of *Penicillium* species used in cheese manufacture. *Journal of Food Protection* 44, 702–710.

Scott, P.M., Kennedy, B.P.C., Harwig, J. and Blanchfield, B.J. (1977) Study of conditions for production of roquefortin and other metabolites of *Penicillium roqueforti*. *Applied and Environmental Microbiology* 33, 249–253.

Shapiro, R., Paster, N., Eyal, O., Menasherov, M., Mett, A. and Salomon, R. (1996) Detection of aflatoxinogenic molds in grains by PCR. *Applied and Environmental Microbiology* 62, 3270–3273.

Smith, J.E and Solomons, G.L. (1994) *Mycotoxins in Human Nutrition and Health*. Directorate General XII, EUR 16048 EN, European Commission, Brussels.

Trail, F., Mahanti, N., Mehigh, R., Liang, S.H., Zhou, R. and Linz, J. (1995a) Physical and transcriptional map of an aflatoxin gene cluster in *Aspergillus parasiticus* and functional disruption of a gene involved early in the aflatoxin pathway. *Applied and Environmental Microbiology* 61, 2665–2673.

Trail, F., Mahanti, N. and Linz, J. (1995b) Molecular biology of aflatoxin biosynthesis. *Microbiology* 141, 755–765.

Waalwijk, C., de Koning, J.R.A., Baayen, R.P. and Gams, W. (1996) Discordant groupings of *Fusarium* spp. from sections *Elegans*, *Liseola* and *Dlaminia* based on ribosomal ITS1 and ITS2 sequences. *Mycologia* 88, 361–368.

Wang, I.K., Reeves, C. and Gaucher, G.M. (1991) Isolation and sequencing of a genomic DNA clone containing the 3 terminus of the 6-methysalicylic acid polyketide synthetase gene of *Penicillium urticae*. *Canadian Journal of Microbiology* 37, 86–95.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Gelfland, D.H., Innis, M.A., Sninsky, J.J. and White, T.J. (eds) *PCR protocols: a Guide to Methods and Applications*. Academic Press, San Diego, pp. 315–322.

Williams, J.K.G., Kubelik, A.R. Livak, K.J., Rafalski, J.A. and Tingey, S.V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* 18, 6531–6535.

Wilson, D.M. (1974) Patulin and penicillic acid. In: Rodricks, J.V. (ed.) *Mycotoxins and Other Fungal Related Food Problems*. American Chemical Society, Washington DC, pp. 90–109.

Yabe, K., Ando, Y., and Hamasaki, T. (1991) A metabolic grid among versiconal hemiacetal acetate, versiconol acetate, versiconol and versiconal during aflatoxin biosynthesis. *Journal of General Microbiology* 137, 2469–2475.

Yu, J., Chang, P.K., Cary, J.W., Wright, M., Bhatnagar, D., Cleveland, T., Payne, G.A. and Linz, J. (1995) Comparative mapping of aflatoxin pathway gene clusters in *Aspergillus parasiticus* and *Aspergillus flavus*. *Applied and Environmental Microbiology* 61, 2365–2371.