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THE APPLICATION OF PCR IN THE DETECTION OF MYCOTOXIGENIC FUNGI IN FOODS

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ABSTRACT

It is estimated that 25 to 50% of the crops harvested worldwide are contaminated with mycotoxins. Because of the toxic and carcinogenic potential of mycotoxins, there is an urgent need to develop detection methods that are rapid and highly specific. The highly advanced physico-chemical methods for the analysis of mycotoxins in use, have the disadvantage that highly sophisticated clean-up and/or derivatization procedures must be applied. An alternative could be the detection of the mycotoxigenic moulds themselves, especially as molecular techniques have been introduced recently as powerful tools for detecting and identifying fungi. PCR methods for the detection of aflatoxigenic *Aspergilli*, patulin-producing *Penicillum* and trichothecene- as well as fumonisin-producing *Fusaria* strains have been described. The usefulness of the PCR methods developed so far to monitor quality and safety in the food an feed industry was already demonstrated. Thus, PCR may be applied to the screening of agricultural commodities for the absence of mycotoxin producers prior to or even after processing. Negative results in this assay indicate that a sample should be virtually free of mycotoxins. Only the positive samples left must be analyzed for the presence of mycotoxins using physico-chemical standard methods. This review does not only summarize the so far developed qualitative and quantitative PCR assays for the detection of mycotoxigenic fungi in agricultural commodities, foods and animal feeds, but describes also strategies to develop new specific PCR assays for such a detection.

Key words: aflatoxin, fumonisin, patulin, polymerase chain reaction, trichothecene.

INTRODUCTION

Nearly every food or feed commodity can be contaminated by fungal organisms and many of the food- and feed-borne filamentous fungi are capable of producing one or more mycotoxins, which are toxic metabolites of concern to both the health of humans and animals (48). While some mycotoxins are produced only by a limited number of species others may be produced by a relatively large range of species from several genera. It is estimated that 25 to 50% of the crops harvested worldwide are contaminated with mycotoxins. The percentage is highest in tropical regions where up to 80% of the crops are reported to contain significant amounts of mycotoxins. Besides crops, foods of animal origin can be contaminated with mycotoxins by carry-over from mycotoxin-containing animal feed. While over 300 mycotoxins have been identified, mostly these have been demonstrated under laboratory conditions and only a relatively small number of about 20 have so far been shown to occur naturally in foods and animal feeds at significant levels and frequency to be of safety concern (Table 1). Because of the toxic and carcinogenic potential of mycotoxins, there is an urgent need to develop detection methods that are rapid and highly specific. Because mycotoxins have diverse chemical structures (Fig. 1), it is not possible to develop one method to detect all relevant mycotoxins, even if some progress was achieved in the simultaneous detection of several mycotoxins (11,54). The highly advanced physico-chemical methods for the analysis of mycotoxins in use, have above all the disadvantage that highly sophisticated clean-up and/or derivatization procedures must be applied (48). In addition, there are much simpler and faster immuno-chemical methods available (48,57), which have in turn the disadvantage to follow the

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Table 1. Mycotoxins relevant to human health.

mycotoxin	generating fungus	contaminated commodity
deoxynivalenol	*Fusarium*	cereals
nivalenol	*Fusarium*	cereals
T2 toxin	*Fusarium*	cereals
fumonisins	*Fusarium*	cereals, legumes, meats, eggs
zearalenone	*Fusarium*	cereals
alternariol	*Alternaria*	cereals, oilseeds
alternariol monomethyl ether	*Alternaria*	cereals
tenuazonic acid	*Alternaria*	cereals, oilseeds
patulin	*Penicillium*	fruits, vegetables, meats, eggs, dairy products
citrinin	*Penicillium*	cereals, fruits, meats, eggs
roquefortine	*Penicillium*	cereals, meats, eggs
penicillic acid	*Penicillium*	cereals
ochratoxin A	*Penicillium*	cereals, tree nuts, spices, dairy products, meats, eggs, fish, coffee
cyclopiazonic acid	*Penicillium*	tree nuts, fruits, dairy products, meats, eggs
compactin	*Penicillium*	tree nuts, fruits, vegetables, fish, dairy products
cyclopaldic acid	*Penicillium*	tree nuts, fruits, dairy products, meats, eggs
citreomycetin	*Penicillium*	fruits, vegetables, meats, eggs
aflatoxins	*Aspergillus*	cereals, peanuts, pistachio, tree nuts, figs, spices, meats, dairy products

concept of "one substance one assay". Therefore, detection of the mycotoxigenic moulds themselves could be an alternative.

At present, monitoring of agricultural commodities, foods and animal feeds for the presence of a fungal contamination includes cultivation and taxonomic identification at the morphological level (29). This approach is, however, very time-consuming, labor-intensive and requires the expertise of mycologists and above all possesses the inherent possibility of misclassification, since morphological characters could be highly variable depending on the media and culture conditions. Therefore, more rapid and more objective methods for the identification of mycotoxigenic fungi in human foods and animal feeds are needed for evaluating the microbiological risks of a given product. Recently, secondary metabolite profiles (12), isoenzyme analysis (10) and molecular techniques (19) have been introduced as powerful tools for detecting and identifying fungi.

NUCLEIC ACID-BASED TECHNIQUES

Nucleic acids present in agricultural commodities, foods and animal feeds are characteristic for the various biological components in complex products. Analysis of specific nucleic acids in these products allows the determination of the presence or absence of certain ingredients or contaminants as well as the identification of specific characteristics of single components (7,32,35). DNA-based detection systems for genetically modified foods (17,21,22,24,25,31,53) and food-borne pathogens (1,2,21,27,51,55), including mycotoxin-producing fungi (6,8,9,18,42,47), have been developed recently. Furthermore, the detection of plant and animal species in the final food products has been shown to be feasible with DNA-based methods (33,34). Because of its high sensitivity, its specificity and rapidity the polymerase chain reaction (PCR) is the method of choice for this purpose (Fig. 2). The PCR is an *in vitro* method to selectively amplify DNA sequences of defined length. This is done by 25 to 40 repetitive cycles of heat denaturation, primer annealing and enzymatic primer extension; thereby generating billions of copies of the DNA sequence in between the primer binding sites. Primers are short single-stranded DNA molecules, usually 18 to 35 bases in length, designed to bind selectively to the complementary sequences of the target DNA segment. During primer annealing one primer has to bind in forward and the other one in a defined distance in reverse orientation to the separated DNA. Therefore, designing PCR primers is a critical step in PCR analysis, since the PCR primers need to have the required sensitivity and specificity.

DEVELOPMENT OF A PCR ASSAY

A general strategy to develop a specific PCR assay to detect mycotoxigenic fungi is given in Fig. 3. For the detection of mycotoxin-producing fungi unique DNA sequences of the respective organisms have to be chosen as primer binding sites. It is concluded that genes involved in the mycotoxin biosynthetic pathway may form a perfect basis for an accurate, sensitive, and specific detection system for mycotoxigenic strains in agricultural commodities, foods and animal feeds, since those genes are supposed to be exclusively present in organisms

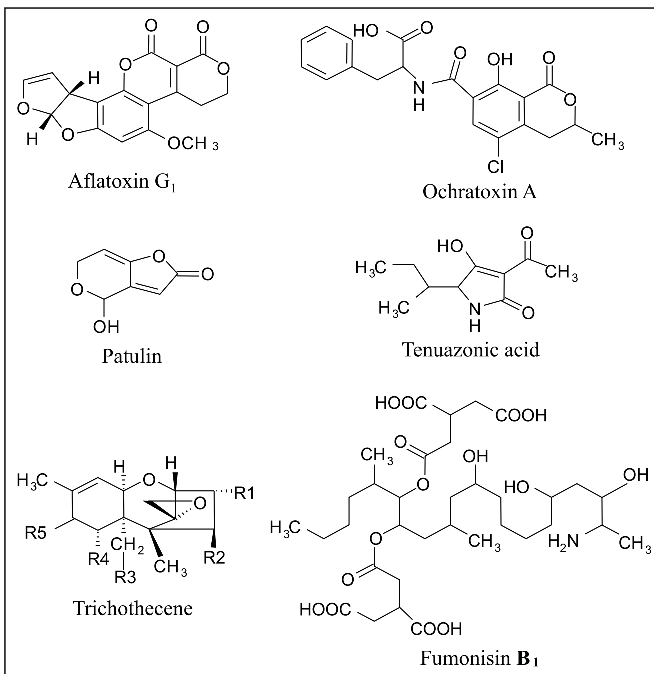

Figure 1. Structures of the major mycotoxins relevant for human health.

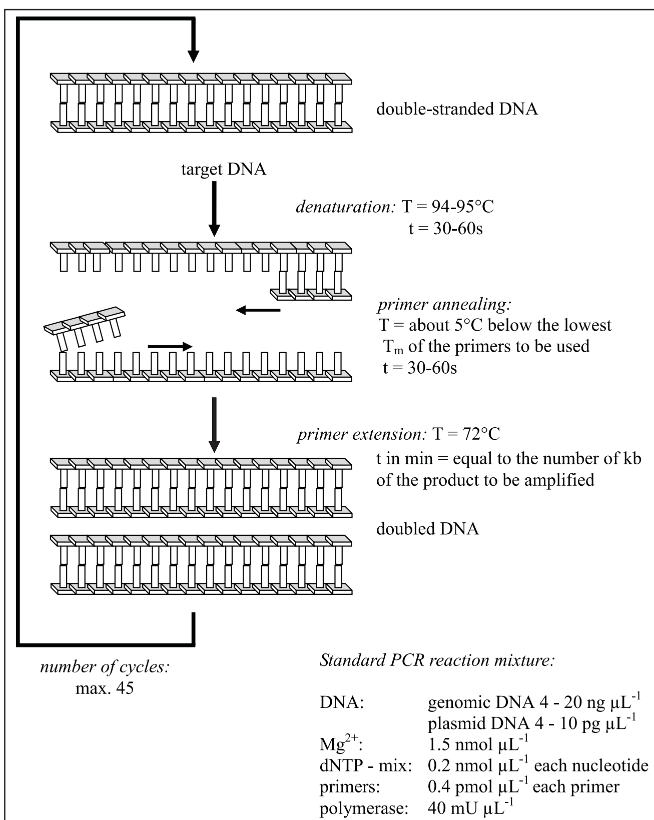

Figure 2. Polymerase Chain Reaction (PCR).

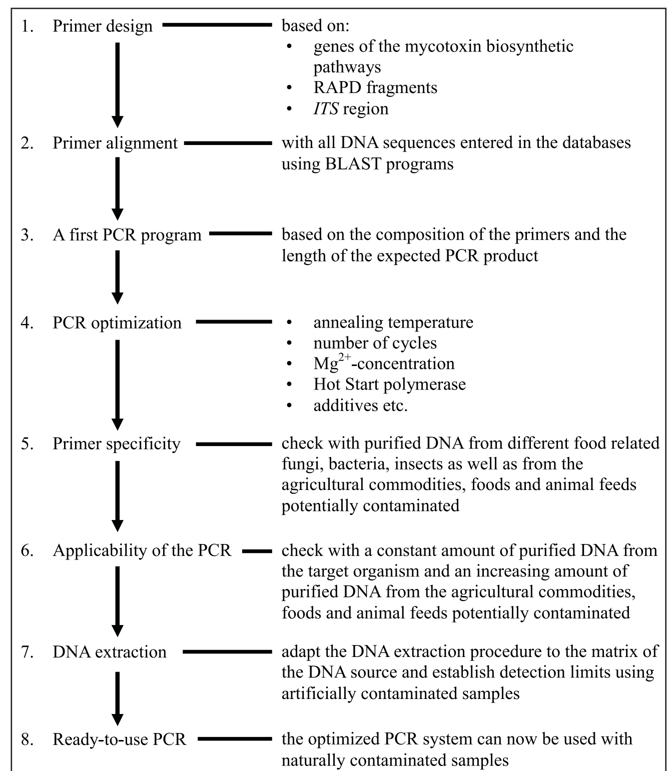

Figure 3. Strategy to develop a specific PCR assay to detect mycotoxigenic fungi.

potentially producing mycotoxins. In the recent years, progress was made to elucidate the biosynthetic pathways for aflatoxins (Fig. 4), patulin (Fig. 5) and trichothecens (Fig. 6) (43,49). Many genes involved in the biosynthesis of these mycotoxins have been identified and their DNA sequences have been published. In the meantime, PCR methods for the detection of aflatoxigenic *Aspergilli* based on the norsolorinic acid reductase encoding gene *nor*1, the versicolorin A dehydrogenase encoding gene *ver*1, the sterigmatocystin O-methyltransferase encoding gene *omt*A, and the regulatory gene *afl*R have been described (8,9,18,47). The trichodiene synthase encoding gene *tri*5, a yet non-identified protein encoding gene *tri*7, and the regulatory gene *tri*6 have been the targets for detecting trichothecene-producing *Fusaria* (6,42). The target sequence for the detection of patulin-producing *Penicillum* strains is within the isoepoxydon dehydrogenase encoding gene *IDH* (43).

If there is only limited or even no information available about genes involved in a certain mycotoxin biosynthetic pathway, specific primers can be designed, for example, by random amplified polymorphic DNA (RAPD) analysis or by analyzing the fungal ribosomal region. Instead of using two primers that are designed based on pre-existing knowledge of the target sequence, RAPDs are produced from single, with respect to sequence randomly chosen, oligonucleotide primers, typically

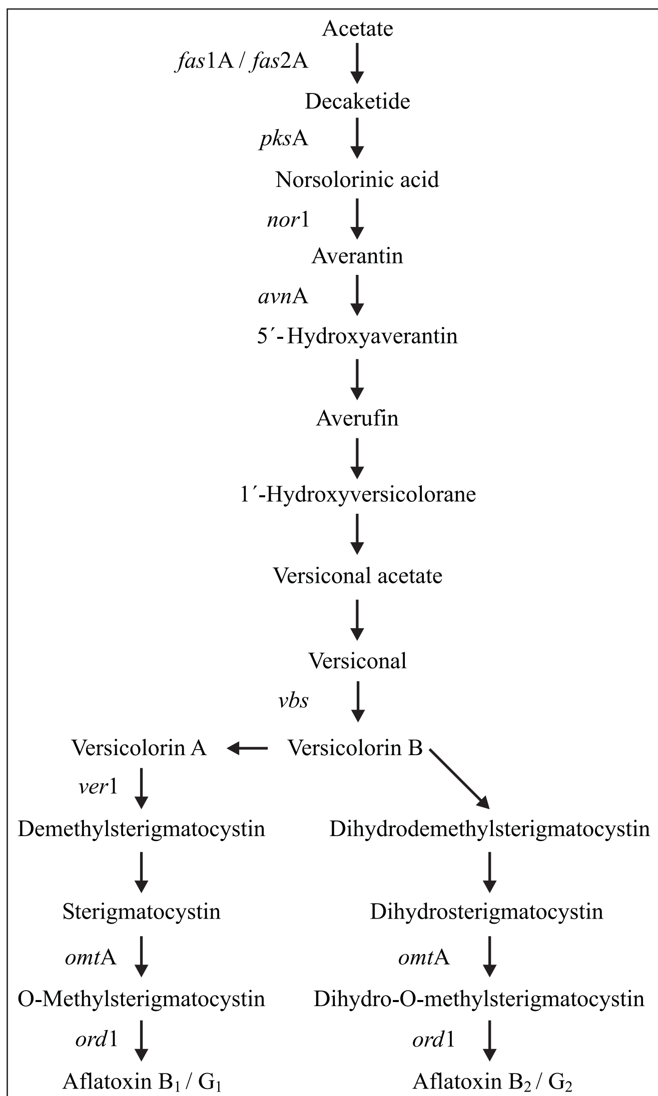

Figure 4. Biosynthetic pathway of aflatoxins.
(*fas*1A/*fas*2A: fatty acid synthase encoding gene; *pks*A: polyketide synthase encoding gene; *nor*1: norsolorinic acid reductase encoding gene; *avn*A: non-identified gene product; *vbs*: versicolorin B synthase encoding gene; *ver*1: versicolorin A dehydrogenase encoding gene, *omt*A: sterigmatocystin O-methyltransferase encoding gene, *ord*1: non-identified gene product; *afl*R: regulatory gene)

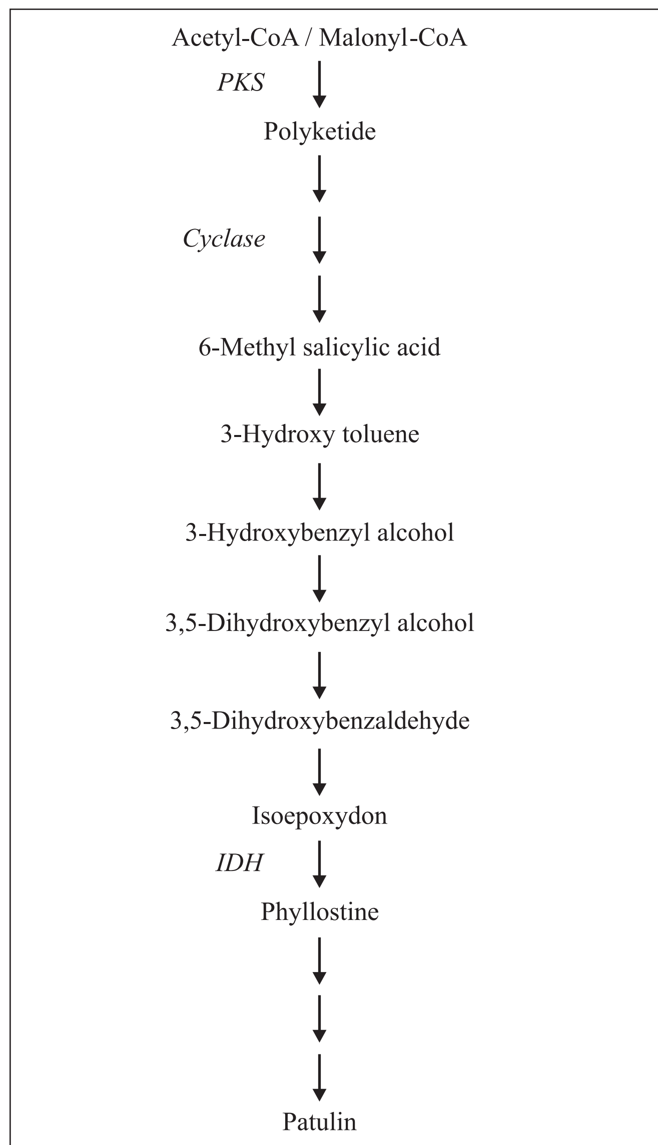

Figure 5. Biosynthetic pathway of patulin.
(*PKS*: polyketide synthase encoding gene; *IDH*: isoepoxydon dehydrogenase encoding gene)

10 bases long. The complexity of eukaryotic nuclear DNA is sufficiently high, that by chance these RAPD primers bind to the DNA in both forward and reverse orientations close enough to one another for PCR amplification. With some randomly chosen RAPD primers no sequences are amplified, with others, the same length products are generated from DNA of different genera, species or subspecies and with still others, patterns of bands are different on genus, species or subspecies level. RAPD fragments unique for the fungi to be detected are sequenced and based on these sequences it should be possible to design primers for the development of a specific PCR system. Möller *et al.* (37) used the UBC18 primer for RAPD analysis of a set of representative *Fusarium* isolates. The strong 600 bp fragment obtained seemed to be exclusively amplified from *Fusarium moniliforme*. The PCR fragment was cloned into a plasmid and sequenced. On the basis of the obtained DNA sequence a specific primer pair for *Fusarium moniliforme* was designed (Table 2). The same approach was used to design primer specific

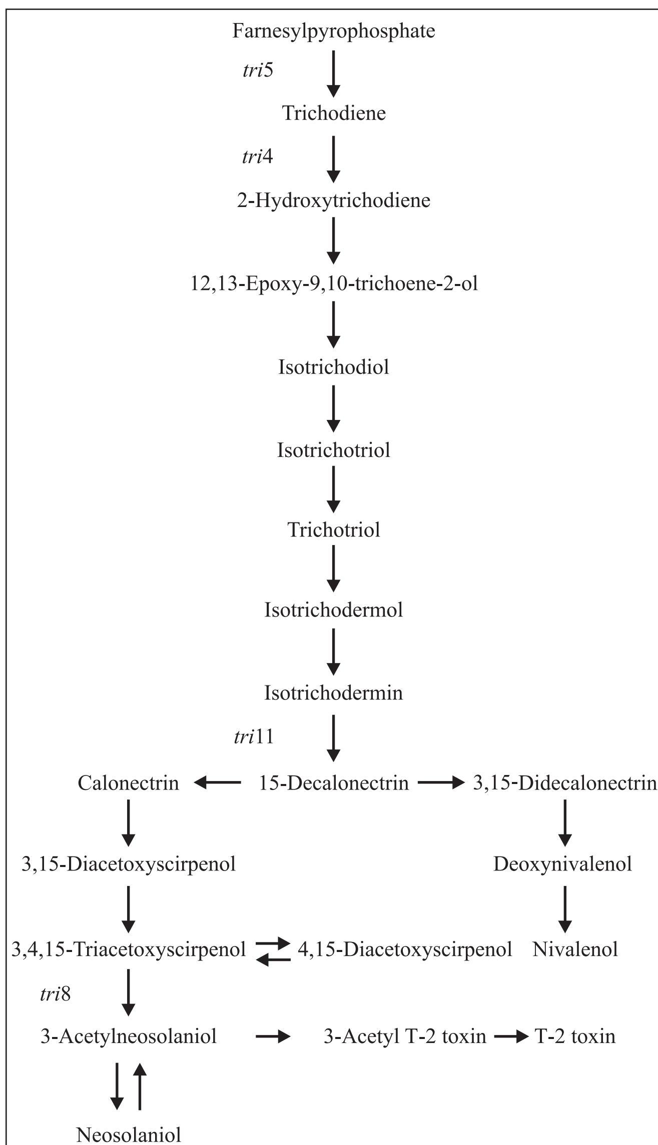

Figure 6. Biosynthetic pathway of trichothecenes.
(*tri*3: non-identified gene product; *tri*4: non-identified gene product; *tri*5: trichodiene synthase encoding gene; *tri*7: non-identified gene product; *tri*8: non-identified gene product; *tri*6: regulatory gene; *tri*11: non-identified gene product)

for *Fusarium subglutinans*, *Fusarium culmorum* and *Fusarium graminearum* (Table 2) (37,40).

The organization of the ribosomal genes is conserved in fungi. Eukaryotic fungal ribosomal genes are arranged in a tandem repeat and within the ribosomal DNA repeat, the two variable non-coding internal transcribed spacer regions (*ITS* regions) are nested between the highly conserved 5.8S nuclear small subunit ribosomal RNA and the two large subunit ribosomal RNA genes. The ribosomal region spanning *ITS*1, 5.8S and *ITS*2 is often between 600 and 800 bp long. The fungal ribosomal genes are highly conserved at the genus level or even higher, but the internal transcribed spacers *ITS*1 and *ITS*2 and the intergenic spacer *IGS* have evolved faster than the ribosomal genes and may therefore be more useful for the development of specific oligonucleotide primers, aimed at differentiating at the genus, species or subspecies level. Several studies have shown that the *ITS* regions are highly variable among and within different fungal species. In order to identify specific primer binding sites for a PCR to detect mycotoxin-producing fungi, either *ITS*1 or *ITS*2 or even both are amplified in several mycotoxin producers and non-producers by using primers binding to the conserved regions of the structural ribosomal RNA genes (40). The sequences of the obtained PCR products are compared in order to identify regions which may serve as target sites in a PCR to distinguish toxigenic from non-toxigenic fungi or to detect fungi at the genus or species level. Grimm and Geisen (23) used *ITS*1 sequences to develop a PCR assay for the detection of potential fumonisin-producing *Fusarium* species (Table 3). In addition, species-specific primers (36) as well as primers for genus level recognition (6) of *Fusarium* species were designed based on the DNA sequence variability found within the internal transcribed spacer regions of the ribosomal DNA (Table 2).

Before setting up a PCR system with the designed primers, the primer sequences should be aligned with all DNA sequences entered in the databases, such as the NCBI sequence database, using BLAST programs to reduce the likelihood of mispriming. Now a PCR assay with the chosen primer pair has to be established using DNA from the target organism isolated from a pure culture as a template. For any given primer pair, a first PCR program can be selected based on the composition of the primers and the length of the expected PCR product (Fig. 2). Sometimes this first PCR program results either in non-specific amplification, that is not only the expected but more PCR products are detected, or in a weak or even no PCR product at all. Then, there is a need to optimize the PCR. Since the aim of PCR is to amplify a specific DNA sequence without any non-specific by-products, primer annealing needs to take place at a sufficiently high temperature to allow only the perfect DNA-DNA matches to occur in the reaction. Thus, the likelihood of non-specific amplification can be reduced by increasing the annealing temperature, whereas decreasing the annealing temperature is a way to improve the yield of the expected PCR product. Within limits, modification in the concentration of magnesium ions may improve the outcome of the PCR assay, and should be considered as a way to optimize PCR. Furthermore, Hot Start is a PCR refinement that suppresses mispriming artefacts and results in a more sensitive, consistent reaction with concomitantly higher yields. The easiest way to carry out a Hot Start is simply to withhold addition of the DNA polymerase until the reaction has reached the denaturation

Table 2. Genus and species-specific primer systems for the detection of mycotoxigenic fungi.

target species	primer designation and sequence		annealing temp. [°C]	PCR product size [bp]	reference
Fusarium species	ItsF	5´-AAC TCC CAA ACC CCT GTG AAC ATA-3´	62	431	(6)
	ItsR	5´-TTT AAC GGC GTG GCC GC-3´			
F. moniliforme	53-6F	5´-TTT ACG AGG CGG CGA TGG GT-3´	65	561	(37)
	53-6R	5´-GGC CGT TTA CCT GGC TTC TT-3´			
F. subglutinans	61-2F	5´-GGC CAC TCA AGA GGC GAA AG-3´	64	445	(37)
	61-2R	5´-GTC AGA CCA GAG CAA TGG GC-3´			
F. culmorum	Fc01F	5´-ATG GTG AAC TCG TCC TGG C-3´	62	570	(40)
	Fc01R	5´-CCC TTC TTA CGC CAA TCT CG-3´			
	175F	5´-TTT TAG TGG AAC TTC TGA GTA T-3´	58	245	(36)
	430R	5´-AGT GCA GCA GGA CTG CAG C-3´			
F. graminearum	Fg16NF	5´-ACA GAT GAC AAG ATT CAG GCA CA-3´	62	280	(40)
	Fg16NR	5´-TTC TTT GAC ATC TGT TCA ACC CA-3´			
	GaoA-V2	5´-AGG GAC AAT AAG TGC AGA-3´	56	896	(26)
	Gao-R2	5´-ACT GTG CAC TGT CGC AAG TG-3´			
F. sambucinum	FSF1	5´-ACA TAC CTT TAT GTT GCC TCG-3´	58	315	(36)
	FSR1	5´-GGA GTG TCA GAC GAC AGC T-3´			
F. oxysporum	FOF1	5´-ACA TAC CAC TTG TTG CCT CG-3´	58	340	(36)
	FOR1	5´-CGC CAA TCA ATT TGA GGA ACG-3´			
F. equiseti	FEF1	5´-CAT ACC TAT ACG TTG CCT CG-3´	58	389	(36)
	FER1	5´-TTA CCA GTA ACG AGG TGT ATG-3´			
F. avenaceum	FAF1	5´-AAC ATA CCT TAA TGT TGC CTC GG-3´	58	314	(36)
	FAR1	5´-ATC CCC AAC ACC AAA CCC GAG-3´			

temperature. This is simple but laborious and a source of contamination, especially when large numbers of samples are analyzed. A better way is to use a modified thermostable DNA polymerase, a so called Hot Start polymerase, which is inactive at room temperature and requires thermal activation. In this case, the activation of the polymerase will occur through incubation of the enzyme for several minutes at 95°C, that is during the first denaturation step. Last but not least, a variety of PCR additives and enhancing agents have been used to increase the yield, specificity and consistency of PCRs. Whilst these additives may have beneficial effects on some amplifications, it is impossible to predict which agents will be useful in a particular context and therefore they must be empirically tested for each combination of template and primers. The most popular additives used are glycerol, dimethyl sulfoxide (DMSO), formamide, betaine, tetramethylammonium chloride (TMAC), bovine serum albumin (BSA) and non-ionic detergents such as Triton X-100, Tween 20 or NP-40.

SPECIFICITY OF THE PCR

After development of an optimized PCR system for the target mycotoxin-producing fungi, the specificity of the chosen primer pairs have to be demonstrated by testing for cross-reactivity against purified DNA from different food-related fungi, bacteria, insects as well as from the agricultural commodities, foods or animal feeds potentially contaminated. The importance to test the primers used for PCR for cross-reactivity was shown clearly by Möller *et al.* (37). They used a primer pair described by Murillo *et al.* (39) to exclusively amplify the DNA from *Fusarium moniliforme* without testing it for cross-reactivity with DNA from related species and it went clear that DNA from other genera

yielded several fragments differing in size and intensity including the predicted amplicon size. Consequently, the PCR assay of Murillo *et al.* (39) is not specific for *Fusarium moniliforme*.

Two very extensive investigation to demonstrate the specificity of the chosen primer pairs can be found in the literature. Niessen and Vogel (42) developed a PCR-based assay to detect *Fusarium* species potentially producing trichothecenes using a pair of primers derived from the DNA sequence of the trichodiene synthase encoding gene *tri*5 (Table 3). The primer pair was tested for cross-reactivity with DNA isolated from a variety of strains representing 64 species and varieties of *Fusarium* as well as from other fungi, bacteria and cereals. Bluhm *et al.* (6) determined the specificity of their *ITS* primers to detect *Fusarium* (Table 2) with purified genomic DNA from 43 fungal species representing 14 genera, including 9 *Aspergillus*, 9 *Fusarium*, and 10 *Penicillium* species. It has been shown that the primers were highly specific for the genus *Fusarium*. All nine *Fusarium* species produced a PCR product of the expected size. In addition, it is of utmost importance to be able to detect the target fungal sequences in a high background of DNA from the agricultural commodity, food or animal feed, since high amounts of non-target DNA may act as a PCR inhibitor and the products to be analyzed may contain only a limited amount of fungi. Therefore, the applicability of a given PCR has to be studied with a constant amount of purified DNA from the target organism isolated from a pure culture with an increasing amount of purified DNA from the agricultural commodity, food or animal feed to be analyzed.

PCR SYSTEMS FOR THE DETECTION OF MYCOTOXIGENIC FUNGI

In the recent years, several detection systems for mycotoxigenic fungi have been developed. PCR methods for the detection of aflatoxigenic *Aspergilli*, patulin-producing *Penicillium* and trichothecene- as well as fumonisin-producing *Fusaria* strains have been described and PCR assays for the detection of ochratoxin-producing fungi are under development.

TRICHOTHECENE-PRODUCING *FUSARIUM* SPECIES

Niessen and Vogel (42) targeted the trichodiene synthase encoding gene *tri*5, which is involved in trichothecene biosynthesis in their group-specific PCR assay for the detection of trichothecene-producing *Fusarium* species. The *tri*5 gene is functionally situated at the very beginning of the trichothecene biosynthetic gene cluster (Fig. 6). The enzyme catalyzes the isomerization and cyclization of farnesyl pyrophosphate to yield trichodiene, the initial specific product in the biosynthetic pathway, leading to the diversity of trichothecene derivatives known. The sequences used for construction of the PCR primers Tox5-1 and Tox5-2 were taken from two highly conserved regions found in the *tri*5 gene showing a distance of 658 bp which is the predicted length of the PCR product (Table 3). Optimization of the PCR cycling program and amplification buffer was performed using a small set of *Fusarium* species. PCR resulted in one single band of the predicted size using the optimized cycling protocol. From the 64 species and varieties of *Fusarium* tested in this study, 21 showed production of an appropriate fragment in the PCR employed. Amplification of a DNA fragment occurred in all species described as producers of trichothecene in the literature reviewed. A missing PCR fragment for some of the species in this study also supports findings by others, which state no production of trichothecene for some species formerly described as producers. No information on the toxigenic potential of *Fusarium lunulosporum* and *Fusarium robustum*, which were *tri*5 positive, could be found in the literature. Their capability to produce trichothecens remains to be studied. Thus, it was demonstrated that the primer pair designed by Niessen and Vogel (42) can be used for a group-specific detection of trichothecene producing *Fusarium* species.

FUMONISIN-PRODUCING *FUSARIUM* SPECIES

A PCR-ELISA for the detection of potentially fumonisin-producing *Fusarium* species has been developed by Grimm and Geisen (23), using the ribosomal *ITS*1 sequence as a target (Table 3). All other ubiquitously occurring food-borne fungi tested showed negative results with this PCR. The fumonisins are a group of mycotoxins produced primarily by *Fusarium* species such as *Fusarium moniliforme*, *Fusarium proliferatum*, *Fusarium nygamai* and *Fusarium napiforme*. Performing the PCR-ELISA increased the specificity of the PCR assay compared to the PCR itself; chromosomal DNA which give false positive results in the PCR were negative after being subjected to PCR-ELISA. All potential fumonisin-producing species gave a positive reaction in the PCR-ELISA and all other species tested were negative. From the data presented in that study, however, the possibility that the PCR-ELISA may cross-react with strains of non-fumonisin producing *Fusarium* species cannot be ruled out. Because of the high variability of the *ITS* regions among and within fungal species, it seems not to be very likely to be able to identify characteristic sequences for the detection of mycotoxigenic fungi within the *ITS* regions. Therefore, the *ITS* regions may not be the best choice for developing a PCR assay to distinguish toxin from non-toxin producers.

TRICHOTHECENE- AND FUMONISIN-PRODUCING *FUSARIUM* SPECIES

Bluhm *et al.* (6) developed a single PCR assay combining the detection of the *Fusarium* genus with the group-specific

detection of both trichothecene-producing and fumonisin-producing *Fusarium* species. Thus, three primer sets were used in this multiplex PCR assay. Primers for genus-level recognition of *Fusarium* species were designed from the *ITS* regions of ribosomal DNA of several *Fusarium* species (Table 2) and the group-specific primer sets were designed from the *tri*6 gene involved in trichothecene biosynthesis and the *fum*5 gene involved in fumonisin biosynthesis (Table 3). Amplification with the *ITS* primers resulted in a PCR product with all *Fusarium* species tested, but did not yield PCR products with any of the non-*Fusarium* species. The three trichothecene-producing *Fusarium* species tested, *Fusarium culmorum*, *Fusarium graminearum*, and *Fusarium sporotrichioides*, all scored positive for the expected product from the *tri*6 primer set and none of the other species tested yielded PCR products. The *fum*5 primer set was also shown to be group-specific for fumonisin-producing *Fusarium* species. Only with *Fusarium verticillioides* and *Fusarium proliferatum* an amplification product of the predicted size was generated.

PATULIN-PRODUCING *PENICILLIUM* SPECIES

A detection system for patulin-producing fungi based on the isoepoxydon dehydrogenase (*IDH*) gene was described by Paterson *et al.* (43). As expected, all patulin-producing fungi tested showed a PCR product, indicating the presence of the *IDH* gene of the patulin biosynthetic pathway (Fig. 5). Unfortunately, some fungi, which do not produce patulin generated a PCR product in the *IDH* PCR, too. Similar problems to distinguish mycotoxin-producers from non-producers by PCR based on one single gene was also observed in the detection of aflatoxigenic fungi.

AFLATOXIN-PRODUCING *ASPERGILLUS* SSPECIES

The use of multiple primer pairs, coding for different stages in mycotoxin biosynthesis, should strengthen the validity of a particular strain's identification as mycotoxigenic. Several multiplex PCR systems for aflatoxin-producing fungi were developed recently (8,9,18,47). Aflatoxins are produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*. Shapira *et al.* (47) described a group-specific assay for the detection for aflatoxin-producing *Aspergillus* species based on the *ver*1, *omt*A, and *afl*R genes (Table 3), which are involved in aflatoxin biosynthesis (Fig. 4), whereas the PCR system described by Geisen (18) targeted the *nor*1, *ver*1 and *omt*A genes (Table 3). In both systems all analyzed aflatoxigenic *Aspergillus flavus* and *Aspergillus parasiticus* strains gave the expected triplet pattern, indicating the presence of all three targeted biosynthetic genes in these strains. Furthermore, the triplet pattern resulting from aflatoxigenic *Aspergillus flavus* strains was identical to that of *Aspergillus parasiticus* strains, indicating high sequence homology between the two species. According to the literature, most of the isolated *Aspergillus parasiticus* strains are capable of producing aflatoxin and should be positive in the multiplex PCR, but only 40 to 50% of the isolated *Aspergillus flavus* strains produce aflatoxin. This inability to produce aflatoxin might be due to deletions or other mutations in the aflatoxin biosynthetic genes. The three non-toxigenic *Aspergillus flavus* strains tested by Geisen (18) showed varying results in the PCR assay. *Aspergillus flavus* BFE310 was negative in all three PCR assays, indicating a deletion of the whole or most of the aflatoxin biosynthetic gene cluster. *Aspergillus flavus* BFE311 was negative with the primer pair specific for the *omt*A gene, indicating a deletion in that gene, whereas *Aspergillus flavus* BFE301 showed the complete triplet pattern, indicating another type of mutation, perhaps in a regulatory gene. Since all genes for aflatoxin production belong to the same gene cluster, it is likely that the deletion of these genes would be the result of a single event. Therefore, it is assumed that in double deletion strains all genes between this two deletion are lost.

The analyzed *Aspergillus versicolor* strain did also possess the same triplet pattern (18), indicating that this strain also contains aflatoxin biosynthetic genes homologous to those of *Aspergillus flavus* and *Aspergillus parasiticus*, respectively. *Aspergillus versicolor* is capable of producing a set of different sterigmatocystins, depending on different ligands or the saturation status of the molecule. Therefore, this PCR result is not surprising, as sterigmatocystin is a direct precursor of aflatoxin and the same enzymes should be needed for its biosynthesis (Fig. 4). Two further species, *Aspergillus oryzae* and *Aspergillus sojae*, which are used as koji moulds for the production of oriental fermented foods, such as sake, miso, soy sauce are closely related to *Aspergillus flavus* and *Aspergillus parasiticus*. Both species do not produce aflatoxin, but the strains studied by Geisen (18) apparently carry sequence homologous to the *ver*1 and *omt*A gene, but the primer set specific for the *nor*1 gene gave no signal indicating missing primer binding sites due to sequence variation in or deletion of that gene. The *nor*1 gene encodes one of the first enzymes within the aflatoxin biosynthetic pathway (Fig. 4). A deletion of this gene or an inactivation due to sequence variation can block the pathway very early and might be an explanation for the inability of the studied strains to produce aflatoxin. In a further study it was shown, that some *Aspergillus oryzae* and *Aspergillus sojae* strains scored positive for all three PCR products (8), but the aflatoxin biosynthetic gene related sequences are pseudo-genes which are not expressed in this strains. Most other food related strains studied showed negative results with all three primer sets (18). The exception was *Penicillium roqueforti* which showed a duplex PCR pattern. The *nor*1 PCR signal had the same length as for the aflatoxigenic *Aspergilli*, whereas the *ver*1 PCR product was slightly larger

than the corresponding amplification product of the aflatoxigenic *Aspergilli*. *Penicillium roqueforti* is capable of producing several polyketide secondary metabolites like patulin, penicillinic acid and mycophenolic acid, but if *Penicillium roqueforti* contains genes similar to the aflatoxin biosynthetic genes of *Aspergillus parasiticus* has to be shown in further studies. Thus, the described multiplex PCRs with three sets of primers were capable of distinguishing *Aspergillus flavus* and *Aspergillus parasiticus* from other food-borne fungi, but not always aflatoxin-producing from non-producing strains of the same species. Therefore, quadruplex PCRs were developed to distinguish aflatoxin-producing from non-producing strains of the *Aspergillus flavus* group. Two quadruplex systems were set up by combining the previously described PCR systems with three primer sets. Therefore, both used the *omt*A, *ver*1, *nor*1 and *afl*R genes as targets. The PCR system developed by Criseo *et al.* (9) uses the PCR primers described by Geisen (18) for the *omt*A, *ver*1 and *nor*1 genes and those described by Shapira *et al.* (47) for the *afl*R gene, whereas the PCR system developed by Chen *et al.* (8) uses the PCR primers described by Geisen (18) for the *ver*1 and *nor*1 genes and those described by Shapira *et al.* (47) for the *omt*A and *afl*R genes (Table 3). In the study of Chen *et al.* (8) 19 strains of the *Aspergillus flavus* group, including *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus oryzae*, *Aspergillus sojae*, and one *Aspergillus niger*, were subjected to PCR testing. Fifteen strains were shown to possess the four target DNA fragments. With regard to aflatoxigenicity, all seven aflatoxigenic strains possessed the four DNA fragments, and five strains bearing less than the four DNA fragments did not produce aflatoxin. On the other hand, all *Aspergillus parasiticus* strains possessed the four DNA fragments, but two of them were non-aflatoxigenic. Similarly, all *Aspergillus oryzae* and *Aspergillus sojae* strains were non-aflatoxigenic, but three of them possessed all four of the DNA fragments. The quadruplex PCR developed by Criseo *et al.* (9) gave comparable results. All aflatoxigenic strains showed a quadruplet pattern, indicating the presence of all four genes of the aflatoxin biosynthetic pathway. Non-aflatoxigenic strains gave varying results with one, two, three or four banding patterns. Thus, the presence of a quadruplet pattern for some non-aflatoxigenic strains of the *Aspergillus flavus* group indicates that this is not a sufficient marker for the differentiation of aflatoxigenic from non-aflatoxigenic strains.

OTHER APPLICATIONS

Furthermore it was shown, that it is possible by PCR to distinguish high from low deoxynivalenol-producing or deoxynivalenol-producing from nivalenol-producing *Fusarium* strains. 17 *Fusarium culmorum* strains studied by Bakan *et al.* (5) produced more than 1 ppm deoxynivalenol (DON) and were considered high-deoxynivalenol-producing strains, whereas 13 *Fusarium culmorum* strains produced less than 0.07 ppm of DON and were considered low-deoxynivalenol-producing strains. According to the *tri*5-*tri*6 sequences, PCR primers were designed in order to distinguish high-producing from the low-producing *Fusarium culmorum* strains (Table 3). Amplification with N1-2 and N1-2R yielded a 200 bp fragment for the high-producing strains, whereas no amplification was observed for the low-producing strains. Conversely, with the 4056 and 3551 primers, amplification yielded a 650 bp fragment for the low-producing strains, whereas no amplification was observed for the high-producing strains. A duplex PCR with both primer sets was also conducted, which resulted in differentiation of the high-producing from the low-producing *Fusarium culmorum* strains in one single multiplex PCR. Lee *et al.* (28) cloned and sequenced the gene cluster for trichothecene biosynthesis from two *Fusarium graminearum* strains, H-11 is a DON producer isolated from maize, and 88-1 is a nivalenol (NIV) producer from barley. Between H-11 and 88-1 all of the *tri* homologues except *tri*7 were conserved, with identities ranging from 88 to 98% and 82 to 99% at the nucleotide and amino acid levels, respectively. The *tri*7 sequences were only 80% identical at the nucleotide level. The *tri*7 genes were aligned and it was found that the *tri*7 open reading frame of H-11 carried several mutations and an insertion containing 10 copies of an 11-bp tandem repeat. The *tri*7 gene from 88-1 carried neither the repeat nor the mutations. A primer pair from the *tri*7 gene was designed to generate a PCR product spanning this *tri*7 insertion (Table 3). 100 *Fusarium graminearum* strains were assayed using this primer pair. Amplification yielded PCR products ranging from 173 to 327 bp, depending on the number of 11-bp repeats within each sequence, from DON-producing *Fusarium graminearum* strains, whereas the PCR-products from NIV-producing strains were with 161 bp identical in size due to a lack of the repeat. The inserted repeats in the *tri*7 sequences varied from 2 to 16 copies for the 50 DON-producing isolates studied.

PCR ASSAY WITH NATURAL SAMPLES

After optimization of the PCR parameters with genomic DNA isolated from the pure target organisms, this optimized PCR can be used with DNA isolated from the products potentially contaminated. Such a PCR analysis includes isolation of DNA from the sample to be analyzed, amplification of the target sequences by PCR, separation of the amplification products by agarose gel electrophoresis and estimation of their fragment sizes by comparison with a DNA length marker after staining with ethidium bromide.

Quality and yield of the isolated DNA are two critical factors in DNA preparations for PCR analysis. The term DNA quality is related to the suitability of the extracted DNA for PCR. Fat, polysaccharides, polyphenols and other secondary compounds are reported to pose a major problem in PCR analysis, since these

Table 3. Group-specific primer systems for the detection of mycotoxigenic fungi.

mycotoxin produced	target gene	primer designation and sequence		annealing temp. [°C]	PCR product size [bp]	reference
trichothecene	*tri*5	Tr5F Tr5R	5´-AGC GAC TAC AGG CTT CCC TC-3´ 5´-AAA CCA TCC AGT TCT CCA TCT G-3´	60	544	(14)
	*tri*5	Tox5-1 Tox5-2	5´-GCT GCT CAT CAC TTT GCT CAG-3´ 5´-CTG ATC TGG TCA CGC TCA TC-3´	68	658	(42)
	*tri*5	HATri/F HATri/R	5´-CAG ATG GAG AAC TGG ATG GT-3´ 5´-GCA CAA GTG CCA CGT GAC-3´	62	260	(15)
	*tri*6	Tri6F Tri6R	5´-CTC TTT GAT CGT GTT GCG TC-3´ 5´-CTT GTG TAT CCG CCT ATA GTG ATC-3´	62	596	(6)
high DON	*tri*5-*tri*6	N1-2 N1-2R	5´-CTT GTT AAG CTA AGC GTT TT-3´ 5´-AAC CCC TTT CCT ATG TGT TA-3´	55	200	(5)
low DON	*tri*5-*tri*6	4056 3551	5´-ATC CCT CAA AAA CTG CCG CT-3´ 5´-ACT TTC CCA CCG AGT ATT TC-3´	55	650	(5)
DON	*tri*7	GzTri7/f1 GzTri7/r1	5´-GGC TTT ACG ACT CCT CAA CAA TGG-3´ 5´-AGA GCC CTG CGA AAG (C/T)AC TGG TGC-3´	60	161+a*11 (a=2,3...16)	(28)
NIV	*tri*7	GzTri7/f1 GzTri7/r1	5´-GGC TTT ACG ACT CCT CAA CAA TGG-3´ 5´-AGA GCC CTG CGA AAG (C/T)AC TGG TGC-3´	60	161	(28)
fumonisin	*ITS*1	Int1 Int2	5´-CCG AGT TTA CAA CTC CCA AA-3´ 5´-ACA GAG TTT AGG GGT CCT CT-3´	65	108	(23)
	*fum*5	Fum5F Fum5R	5´-GTC GAG TTG TTG ACC ACT GCG-3´ 5´-CGT ATC GTC AGC ATG ATG TAG C-3´	62	845	(6)
aflatoxin	*afl*R	aflR660 aflR1249	5´-CGC GCT CCC AGT CCC CTT CAT T-3´ 5´-CTT GTT CCC CGA GAT GAC CA-3´	59	630	(49)
	*afl*R	APA-450 APA-1482	5´-TAT CTC CCC CCG GGC ATC TCC CGG-3´ 5´-CCG TCA GAC AGC CAC TGG ACA CGG-3´	65	1032	(46)
	*ord*1	ord1501 ord2226	5´-TTA AGG CAG CGG AAT ACA AG-3´ 5´-GAC GCC CAA AGC CGA ACA CAA A-3´	58	719	(49)
	*nor*1	nortaq-1 nortaq-2	5´-GTC CAA GCA ACA GGC CAA GT-3´ 5´-TCG TGC ATG TTG GTG ATG GT-3´	55	66	(4,30)
	*nor*1	nor1 nor2	5´-ACC GCT ACG CCG GCA CTC TCG GCA C-3´ 5´-GTT GGC CGC CAG CTT CGA CAC TCC G-3´	65	400	(18)
	*ver*1	ver1 ver2	5´-GCC GCA GGC CGC GGA GAA AGT GGT-3´ 5´-GGG GAT ATA CTC CCG CGA CAC AGC C-3´	65	537	(18)
	*ver*1	VER-496 VER-1391	5´-ATG TCG GAT AAT CAC CGT TTA GAT GGC-3´ 5´-CGA AAA GCG CCA CCA TCC ACC CCA ATG-3´	65	895	(46)
	*omt*A	omt1 omt2	5´-GTG GAC GGA CCT AGT CCG ACA TCA C-3´ 5´-GTC GGC GCC ACG CAC TGG GTT GGG G-3´	65	797	(18)
	*omt*A	OMT-208 OMT-1232	5´-GGC CCG GTT CCT TGG CTC CTA AGC-3´ 5´-CGC CCC AGT GAG ACC CTT CCT CG-3´	65	1024	(46)
patulin	*IDH*	IDH-1 IDH-2	5´-CAA TGT GTC GTA CTG TGC CC-3´ 5´-ACC TTC AGT CGC TGT TCC TC-3´	52	600	(43)

compounds can irreversibly interact with proteins and nucleic acids and may therefore act as PCR inhibitors (13,44). Depending on the quality of the DNA template and the primer systems used, the detection limits of the PCR with DNA isolated from pure fungal cultures on agarose gels stained with ethidium bromide were reported to be 1 to 10 pg for multicopy genes (6) and 5 to 1000 pg for single copy genes (20,37,40), the latter corresponds to 100 to 20,000 fungal genome equivalents. Detection limits in foods were determined to be 10 to 100-fold higher. Furthermore it was reported, that PCR even fails with food samples due to the presence of PCR inhibitors. Therefore it has to be shown, that a developed PCR system is still working with DNA extracted from complex food matrices. Shapira *et al.* (47) failed to detect aflatoxigenic fungi on maize even at the highest inoculum levels of 10^6 spores per gram of maize by direct extraction of the DNA from the maize samples. By including an 12h enrichment step, this problem could be overcome, but at the expense of the time needed for detection. Since enrichment steps result in the dilution of inhibitors, in an increased number of target cells and in an increased ratio of DNA derived from viable to DNA derived from dead cells, an improvement of the sensitivity of the detection system as well as a detection of only viable cells is achieved. The detection of only viable cells is of utmost importance in the detection of pathogenic microorganisms such as *Salmonella* or *Listeria*, because only viable cells have the potential to be toxigenic. For the evaluation of a potential contamination with mycotoxins however, it is an advantage to be able to detect a current as well as a former contamination with mycotoxigenic fungi. Because of their high stability, a given mycotoxin may persist in a critical concentration in a product even when the generating fungus is no longer present.

DNA ISOLATION

The amount and quality of the extracted DNA depends largely on the combination of sample matrix and extraction method applied. Therefore, a unique DNA isolation procedure for all the different products which have to be analyzed does not exist. To adapt the extraction procedure to the matrix of the DNA source and to establish the detection limits of the studied systems artificially contaminated samples have to be used. The identification of products as naturally contaminated with mycotoxigenic fungi could then be performed by using the optimized PCR assay, that is DNA isolation and the PCR itself. In principle, two different DNA isolation protocols with plenty of variations are used for DNA extraction from complex food matrices. The classical protocol for DNA isolation is based on an incubation of the samples in the presence of a detergent such as cetyltrimethylammonium bromide (CTAB) or sodium dodecylsulfate (SDS) and a treatment with organic solvents such as chloroform and phenol, respectively, followed by precipitation of DNA with isopropanol or ethanol (58). The second protocol is based on commercially available DNA-binding resins as ready to use kits. In general, the classical detergent-based methods result in higher DNA yields but poorer DNA quality compared to the DNA-binding resins and the extraction is more time-consuming (21,58).

The yield and purity of the extracted DNA could be improved by adapting the extraction procedure to the matrix of the DNA source and the results presented in the literature demonstrate the functionality of the developed PCR assays to detect mycotoxigenic moulds without an enrichment step by isolation of DNA directly from agricultural commodities, foods or animal feeds, such as maize (6), wheat (26), peanuts (8), and figs (16). In general, the sample material was frozen in liquid nitrogen, ground in a mortar and re-suspended in a lysis buffer. An improvement of the sensitivity of the PCR assays has been shown by exclusion of inhibitory compounds from natural samples. One approach is to increase the ratio of fungal to sample tissue by using only the surface area of the sample tissue for DNA extraction (16). The other approach is to enhance fungal cell wall disruption prior to DNA extraction. This was achieved by thoroughly mixing the sample material with alumina in a lysis buffer (4,16,20). As with the liquid nitrogen method, in some PCR assays additional bands appeared and the specific PCR products were rather weak (16,20). These artefacts might be due to impurities from the sample tissue and could influence the interpretation of the results. The occurrence of artificial bands was greatly reduced with template DNA isolated by the ultrasonic method (38), whereby the samples were sonicated in a lysis buffer for 1 minute at maximum amplitude. Due to the rapidity and the possibility to handle multiple samples, the ultrasonic approach is also suitable for routine analysis. For purification of the extracted DNA the DNeasy Plant Mini kit from Qiagen has been widely used (8,20,26,38,45).

CONTROL REACTIONS

Once the primers are designed and the conditions for a robust assay are optimized, PCR is a very sensitive, rapid, and relatively easy to handle assay for the detection of mycotoxigenic fungi in agricultural commodities, foods and animal feeds. The total detection time, that is the period of time from when the samples were taken until the PCR products were visualized, is approximately 7 h. To exclude false positive and/or false negative results in the PCR analysis several controls have to be included into the methodology (Fig. 7). To exclude false negative results, the absence of inhibitors has to be shown by a control PCR using an internal standard or by spiking the DNA preparation with the target sequence. It is also possible to check DNA quality by a separate PCR using a target sequence always present in the product to be analyzed. For plant-based materials conserved regions of the 18S ribosomal RNA gene could be chosen as primer binding sites. Since such a primer pair generates a PCR product with plant as well as fungal DNA, a missing

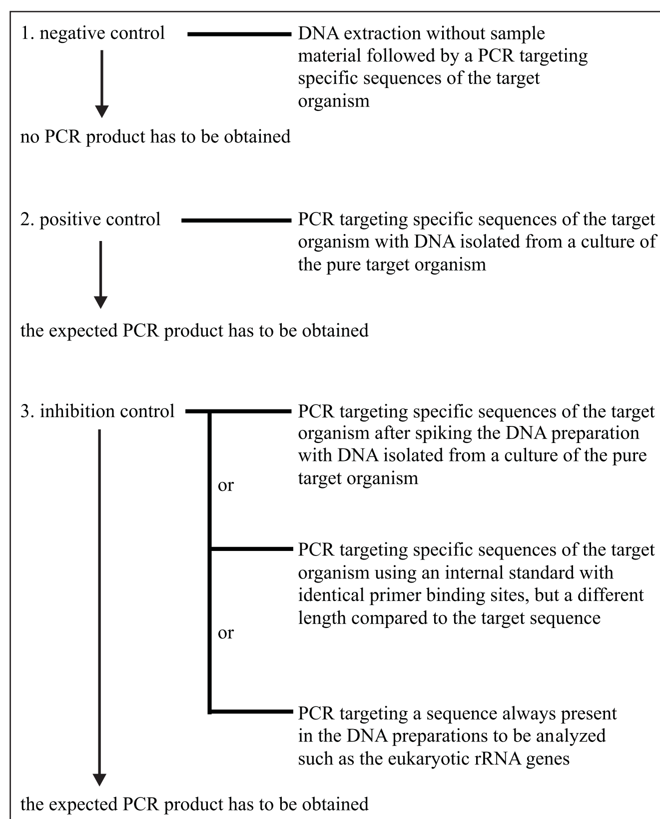

Figure 7. Control reaction which have to be included into a PCR assay to detect mycotoxigenic fungi.

amplification product in the above mentioned control reaction points either to PCR inhibitors or poor DNA quantity in the DNA preparations. False negative results might also be due to the inactivation of PCR reagents. To assure the quality of the PCR reagents, a positive control is used, that is the quality of the PCR reagents is tested by performing a PCR close to the limit of detection with pure target DNA. To avoid misinterpretation due to false positive results because of a contamination of reagents or the laboratory itself, a negative control is applied, that is performing the complete PCR assay from extraction to amplification without a DNA source.

DETECTION OF PCR PRODUCTS

One of the most frequently used methods for the detection of PCR products is agarose gel electrophoresis followed by staining with fluorescent dyes such as ethidium bromide or SYBR® Green, which fluoresce upon intercalation into the DNA double strand. A potentially mycotoxigenic fungi is supposed to be detected, if an amplification product of the predicted size is obtained. A verification of the PCR result is highly recommended, because sometimes false positive results have been observed, that is an amplification product not derived from the target sequence but, because of a very similar fragment length not distinguishable from the expected amplification product by agarose gel electrophoresis. To verify the PCR results, restriction analysis, that is specific cleavage of the amplification product by restriction endonucleases, or Southern blotting followed by hybridization, that is transfer of the amplification product onto a membrane followed by hybridization with a DNA probe specific for the target sequence, may be used. Alternatively amplification products may be verified by direct sequencing or a second nested or semi-nested PCR.

Appropriate technical equipment is recommended for detection of PCR products by agarose gel electrophoreses followed by staining with DNA dyes and the work is labor-intensive and time-consuming. Furthermore, DNA dyes are hazardous for humans as they are potential mutagens. Therefore, novel system for a more rapid and convenient detection of PCR products have been developed (26,36,41). One of the systems is based on a PCR in which one of the primers is biotinylated and digoxigenin-11-dUTP is incorporated during elongation (41). Biotinylated PCR products are captured on streptavidin-coated solid supports, and alkaline phosphatase conjugated to anti-digoxigenin antibody is subsequently bound to the incorporated digoxigenin. The detection may be obtained with colorimetric, fluorescent, or luminescent substrates for alkaline phosphatase. The detection system can be performed in microtiter plates allowing easy handling of multiple samples. The total assay time following the PCR is about 1h dependent on the type of substrate and the type of solid support applied in the system.

Knoll *et al.* (26) adopted a new commercially available test strip system to set up a fast, sensitive and easy to handle method for the detection of *Fusarium graminearum* contamination in cereal samples. DNA Detection Test Strips™ were used for PCR product detection and the method was compared to agarose gel electrophoresis. Detection of PCR products was performed in only 20 min without the need of special technical equipment or hazardous fluorescent DNA dyes. A 5'-digoxigenin labeled primer pair targeting the galactose oxidase was used for PCR (Table 2). Hybridization of the PCR product was performed with a biotin labeled oligonucleotide probe specific for the DNA sequence of the PCR product. As an advantage over detection of a PCR fragment in agarose gels, the Test Strips™ provide both detection and identification. Even in the case of non-specific amplification, products which do not hybridize to the specific probe will not be detected, but in contrast to an agarose gel, the size of the PCR product can not be determined. Therefore, the use of DNA Detection Test Strips™ is only recommended if a completely optimized PCR protocol exists, in which it was already shown that the desired PCR product is generated, if target DNA is present in sufficient amounts. The limit of detection was found to be 260 pg template DNA per reaction with the Test Strip™ system. This is slightly higher in comparison to the use of gel

electrophoresis for amplification product detection. In the agarose gel, a clearly visible signal could still be seen at a concentration of 65 pg template DNA and at a concentration of 16.3 pg DNA a faint band was still detectable.

Mishra *et al.* (36) developed a fluorescent-based PCR assay that allows a rapid and reliable identification of five toxigenic and pathogenic *Fusarium* species without the need of using hazardous fluorescent DNA dyes such as ethidium bromide. The species include *Fusarium avenaceum*, *Fusarium culmorum*, *Fusarium equiseti*, *Fusarium oxysporum* and *Fusarium sambucinum* (Table 2). The method is based on the PCR amplification of species-specific DNA fragments using fluorescent oligonucleotide primers, which were designed based on sequence divergence within the internal transcribed spacer region of nuclear ribosomal DNA. All the upstream primers were conjugated at their proximal end with different fluorescent dyes. The fluorophores were chosen so as to have minimal spectral overlap and can be excited with ultraviolet light. The five *Fusarium* species could be differentiated from each other on the basis of a single PCR amplification with high confidence and precision. Species-specific fluorescent PCR primers amplified an expected size DNA fragment only from the isolates of *Fusarium* species from which the primer was originally designed. The resulting color was clearly visible after exciting the electrophoresed agarose gel with ultraviolet light. The technique was further modified to preclude the need for gel electrophoresis. The PCR products were purified using Qiagen PCR Purification Kit to remove excess, unused and non-conjugated dye, and unused primers. Subsequently, the amplified color was visualized under ultraviolet light in reaction tubes, which were photographed with a hand-held Polaroid camera.

RNA-BASED APPROACH

It has to be kept in mind that the absence of a PCR band does not necessarily imply that the analyzed strain is unable to produce mycotoxins. For example, a mutation in the primer binding site not affecting the functionality of the encoded protein could be the reason for a missing PCR product. In addition, the generation of the expected PCR product does not necessarily imply that the analyzed strain is capable of producing mycotoxins, because the presence of a PCR product does not allow to draw any conclusion about expression of the respective gene. However, the presence or lack of mRNAs may permit direct differentiation between mycotoxin-producing and non-producing strains. Although Northern hybridization analysis and RNAse protection assays are well established and reliable techniques for the detection of mRNAs, their sensitivity may be insufficient for the detection of low levels of gene transcription. Therefore, detection systems based on reverse transcription PCR (RT-PCR) have been developed to monitor aflatoxin production in *Aspergillus parasiticus* (50) and trichothecene production in *Fusarium culmorum* (14). RT-PCR has been demonstrated to be 100-1000 times more sensitive than Northern hybridization analysis. In addition, several specific mRNAs may be detected simultaneously in a single RNA sample by multiplex RT-PCR. RT-PCR allows the detection of mRNAs transcribed by specific genes by the PCR amplification of cDNA intermediates synthesized by reverse transcription. Sweeney *et al.* (50) designed two pairs of oligonucleotide primers from the coding regions of the structural gene *ord*1, which encodes a cytochrome P450 monooxygenase involved in the conversion of the penultimate aflatoxin pathway intermediate O-methylsterigmatocystin to aflatoxin B1, and the intronless regulatory gene *afl*R that positively regulates the transcription of the aflatoxin biosynthetic genes (Fig. 4, Table 3). Total RNA was used as a template to synthesize cDNAs with random hexamer primers and reverse transcriptase. The resulting cDNA was amplified by PCR using the specific primers. Since genomic DNA sequences homologous to RNA targets present in the PCR reaction may interfere with the detection of RNA by RT-PCR, the RNA preparations were treated with DNAse I first. As a control, the gene transcription of a housekeeping gene, ß-tubulin, was monitored by RT-PCR. To distinguish cDNA from genomic DNA targets, the *ord*1 and ß-tubulin primers were designed from coding regions flanking introns, so that the decreased size of the RT-PCR product relative to the PCR product derived from genomic DNA could be discerned. This strategy allows the detection of false positive reactions resulting from contamination of the RNA treated with DNase I with genomic DNA from foreign cellular material or carry-over contamination from previous PCR reactions and also assessed the efficiency of the DNase I treatment of the isolated RNA. The developed RT-PCR was successfully used to distinguish aflatoxin-producing from non-producing *Aspergillus parasiticus* strains, but this technique has also the potential to be employed as a tool to investigate the effects of a variety of physiological factors on the transcription of the aflatoxin genes. In addition, the RT-PCR primers designed from aflatoxin genes of *Aspergillus parasiticus* may also be employed to monitor the transcription of aflatoxin genes of *Aspergillus flavus* as aflatoxin gene homology is very high between these two aflatoxigenic species.

Doohan *et al.* (14) developed a RT-PCR assay to successfully study the expression of the *tri*5 gene involved in trichothecene biosynthesis in *Fusarium* species (Fig. 6, Table 3). Quantification of *tri*5 gene expression was performed relative to expression of ß-tubulin, which has been used previously as a measure of mRNA extraction efficiency and fungal biomass. The ß-tubulin and *tri*5 primer sets both flanked introns within the respective genes, which allowed size differentiation of amplified genomic DNA and mRNA. Quantification of ß-tubulin expression in RNA extracts obtained in a time course experiment in which genomic DNA was used as a competitor template showed, that

ß-tubulin expression was constant over time. ß-Tubulin was used to normalize the *tri*5 expression results and thus accounted for any variability arising from the RNA quantification or RT step. *Tri*5 expression at 24, 48, 72 and 96 h post-inoculation was quantified relative to expression of ß-tubulin, and the DON contents of culture supernatants were determined. Under these conditions, a direct relationship between *tri*5 expression and the increase in DON production over time was observed.

QUANTITATIVE PCR

Compared to DNA, however, RNA is relatively unstable. This is largely due to the presence of ribonucleases (RNases), which break down RNA molecules. RNases are very stable, do not require cofactors, are effective in very small quantities, and are difficult to inactivate. Isolation and analysis of RNA therefore requires specialized techniques. This situation is even more complicated by the fact that changes in gene expression can occur during handling of the sample and isolation of the RNA. To avoid the need of RNA isolation, it was investigated, if the amount of PCR product generated from a DNA target correlates with fungal biomass or even mycotoxin concentration of a given sample. Niessen and Vogel (42) used the Tox5 PCR assay (Table 3) to analyze samples of wheat for the presence of trichothecene-producing *Fusarium* species and they found that the intensities of the PCR bands were well correlated with the concentration of DON detected by HPLC in the same wheat samples. This implies that there is a quantitative correlation between toxin production and biomass in naturally contaminated materials, which was denied by most studies performed in the past. Since different numbers of target molecules in the samples used for PCR may result in identical numbers of the amplification product in the plateau phase and identical numbers of target molecules in different numbers of the amplification product, it is, in general, not possible to conclude from the numbers of the PCR product generated in a standard PCR to the numbers of target molecules originally present in the samples used for PCR. Therefore, quantitative PCR systems have been developed which do not use end point measurement to quantify the amount of target molecules present in the sample, such as the real time PCR systems, or in which such a end point measurement does not pose problems for quantification, such as competitive PCR.

COMPETITIVE PCR

The principle of the competitive PCR is a co-amplification of an increasing amount of target DNA with a defined amount of a competitor, that is an internal DNA standard. Therefore tube-to-tube differences and inhibitors do not interfere, because their effect on target and competitor is identical. To guarantee nearly identical amplification efficiencies of both the target and the competitor sequence, the same primer binding sites are used to amplify the target DNA and the competitor. Both amplification products should be distinguished from each other by a difference in fragment length. Therefore, the competitor is constructed by introduction of short deletions or insertions into the target sequence. Some studies have indicated that the use of homologous competitors may lead to heterodimer formation during PCR. For this reason, heterologous competitors were generated, which had 5´ and 3´ termini identical to the fungal target primer binding sites, but which had no internal sequence homology to the target sequence. This approach, however, may result in different amplification efficiencies of the target and competitor sequences. Since the competitor is used as an internal DNA standard it acts also as an internal amplification control. For quantification, a calibration curve with a serially diluted solution of pure fungal genomic DNA has to be generated with every experiment. In general, a log-log plot of PCR product ratio against fungal DNA concentration gives a linear regression. PCR product ratios were determined after densitometric gel evaluation by dividing the band intensity of the target DNA product by that of the competitor product. Nicholson *et al.* (40) developed a competitive PCR assay based on RAPD primers to detect and quantify *Fusarium culmorum* and *Fusarium graminearum* in cereals (Table 2). Competitive PCR indicated that the barley and wheat samples studied are contaminated with different levels of *Fusarium culmorum* or *Fusarium graminearum*, respectively, but no approach was made to correlate this amount of fungal DNA with pathogen biomass.

A PCR-based assay to quantify trichothecene-producing *Fusarium* based on primers derived from the trichodiene synthase encoding gene *tri*5 was developed by Edwards *et al.* (15) (Table 3). Regression analysis of trichothecene-producing *Fusarium* and DON in harvested grain from an inoculated-field trial showed a good correlation between *tri*5 DNA and the amount of DON produced. This relationship will probably be weaker for field samples, which may differ in the trichothecene-producing isolates present. Those isolates may differ in their capability to produce DON or, in the case of *Fusarium graminearum*, in the relative amounts of DON and NIV that they produce.

REAL TIME PCR

Competitive PCR needs little capital investment and little expenditure on consumables, but, on the other hand, competitive PCR is time-consuming and labor-intensive. In contrast, real time PCR systems are more convenient and more rapid, but expensive thermocyclers equipped with a fluorescence reader are needed. Real time PCR enables calculation of the amount of PCR product present in a sample at a point of the reaction in which strict exponentiality of DNA amplification is given. The so called LightCycler™- and TaqMan™-technology are mainly

used in the detection and quantification of mycotoxigenic fungi. Both methods are well suited for automation and high throughput of samples.

LIGHTCYCLER™ PCR

The LightCycler™-system makes use of performing PCR in a small reaction volume in a glass capillary exposed to a temperature regulated air stream, which results in very rapid thermal cycling protocols. A PCR with 35 cycles is completed within about 25 min, but due to the LightCycler™ only 32 samples can be run at the same time. For quantitative PCR, a fluorescence technique based on SYBR® Green dye was applied. The dye possesses selective affinity to double stranded DNA and the binding greatly enhances fluorescence emission of the molecule at 530 nm. SYBR® Green does not react with single stranded DNA or with RNA present in the sample. Intensity of fluorescence at 530 nm is proportional to the concentration of double stranded DNA and thus providing a measure for the quantity of newly synthesized product during PCR. For quantification, a calibration curve with a serially diluted solution of pure fungal genomic DNA has to be generated with each set-up of the real time PCR. A drawback of this method might be the unspecific binding of SYBR® Green to double stranded DNA in which case false positive reactions may result. Therefore, amplification products are characterized by melting point analysis. Non-specific products should be distinguished from the desired PCR product by a difference in the melting points.

Bagnara *et al.* (4) developed a SYBR® Green quantitative PCR system based on the *nor*1 gene to detect and quantify aflatoxigenic *Aspergillus flavus* in black pepper (Table 3). The minimal cell number which can be detected by this system was determined to be 4.5 x 10^3/g of pepper. Non-infected pepper gave always negative results both with the PCR approach as well as with plate counting. With infected pepper, the initial *nor*1 copy numbers were always higher than the spore numbers obtained by plate counting and it was not possible to correlate the *nor*1 copy numbers with the spore numbers. In general both values increased with prolonged incubation time, but the ratio of the *nor*1 copy numbers to the spore numbers decreased steadily from about 5 x 10^3 after 5 days of incubation to only about 15 after 15 days of incubation.

A SYBR® Green quantitative PCR system based on the *tri*5 gene to detect and quantify trichothecene-producing *Fusarium* species in cereal samples was developed by Schnerr *et al.* (45) (Table 3). The detection limit of the PCR system was determined to be below 50 pg of fungal DNA. The developed LightCycler™ PCR assay provided a highly reproducible measurement, since an average coefficient of variation of 2% over the whole concentration range, with the highest variation at the lower concentrations, was found. The sensitivity of the PCR was greatly improved by Hot Start technique and the use of 1 unit of pyrophosphatase per amplification reaction resulted in a 10% improvement in PCR product yield. The application of uracil DNA glycosylase prevented carry-over of contaminating PCR products. Uracil DNA glycosylase has no effect on native DNA, because native DNA does not contain uracil and in addition, the enzyme is inactivated by heat denaturation prior to PCR, in which deoxythymidin triphosphate (dTTP) has to be substituted by deoxyuracil triphosphate (dUTP). The developed quantitative LightCycler™ PCR-based assay was used to quantify the DNA from trichothecene-producing *Fusarium* species in 300 field inoculated wheat samples (46). The minimum detectable quantity of template DNA was 16 ng per gram of sample corresponding to 290 gene copies. Data analysis revealed a positive, linear correlation with a coefficient of correlation of r=0.9557 between DON concentration and DNA amounts over all samples. This correlation was demonstrated to be highly significant. If only the samples with DON concentrations below 1.5 mg/kg were analyzed, a correlation r=0.7476 between DON concentration and DNA amounts were calculated. An interval of confidence for P=95% was calculated based on samples with DON concentrations below 1.5 mg/kg. Only 12 of 234 samples had data points which were outside the calculated interval. As an example, the range of probable DON concentration at a given DNA content of 0.5 mg/kg was calculated to be 0.74±0.57 mg/kg. This means, a sample with this concentration of *Fusarium* DNA will display a DON concentration between 0.17 and 1.31 mg/kg with a probability of 95%. Divergence from such a correlation may be explained by the fact that the PCR measures the total *Fusarium* biomass in the samples, whereas the microbiological technique applied measures the percentage of kernels infected, rather than biomass. In addition, only living mycelia is detected by plate counting. Therefore, the developed LightCycler™ PCR is a suitable tool, because it provides a measure for the determination of biomass changes over time, taking in account living mycelia actively producing DON but also dead mycelia, which formerly had contributed to the DON content actually found.

TAQMAN™ PCR

The TaqMan™-technology makes use of the 5´-3´ exonuclease activity of the polymerase to generate a template-specific fluorescent signal after hydrolyzing an internal probe during each step of the PCR. The internal probe is 5´-labeled with a reporter fluorescent dye and 3´-ligated to a quencher dye. As long as the reporter fluorescent dye and the quencher dye are located in close proximity on the internal probe, the quencher dye greatly reduces the fluorescence emitted by the reporter dye by Förster energy resonance transfer (FRET) through space. During PCR, the reporter dye is separated from the quencher dye, which results in an increase of the reporter dye signal. Only if the internal probe is binding to the DNA in

between the two PCR primers a fluorescence signal during PCR is generated. Therefore, this additional hybridization step increases the specificity of the PCR. False negative results due to PCR inhibitors could be excluded by using an internal amplification control in each PCR. The parameter measured is the threshold-cycle (CT) where each reaction trespasses a certain fluorescence level. In comparison to the LightCycler™ PCR, the TaqMan™ PCR needs more time, but on the other hand, more samples can be run at the same time. A PCR with 45 cycles is completed within about 2 h with at maximum 96 single reactions per run. For quantification, a calibration curve with a serially diluted solution of pure fungal genomic DNA has to be generated with each set-up of the real time PCR.

A TaqMan™ quantitative PCR system directed against the *nor*1 gene of the aflatoxin biosynthetic pathway as a target sequence has been developed by Mayer *et al.* (30) (Table 3). The PCR was applied to detect an aflatoxigenic *Aspergillus flavus* strain in artificially contaminated plant-type foods like maize, pepper and paprika. In general, a good correlation between the spore numbers, determined by microscopic counting, and the *nor*1 gene numbers was observed, however, compared to the spore numbers, the *nor*1 copy numbers were always higher. The detection limit of this PCR system was determined to be about 10^3 cells per gram for the pepper and somewhat higher for the maize and paprika samples. It was suggested that with low fungal DNA concentrations an inhibition takes place, apparently by competition through high amounts of unspecific plant DNA. The general observation that the target gene copy numbers determined by quantitative real time PCR are higher than the spore numbers determined by plate counting might be due to the recording of viable and dead cells by PCR, but not by plate counting as well as the presence of mycelial fragments in the samples to be analyzed. Mycelial fragments will give rise to only one colony, even if they consist of many cells. In addition, mycelial cells carry several nuclei and each nucleus contributes to the number of target gene copies. The target gene copy numbers were reported to be 1.2 to 3.5 log units higher than the spore numbers.

CONCLUSIONS AND FUTURE PERSPECTIVES

PCR may be applied to the screening of agricultural commodities for the absence of mycotoxin producers prior to or even after processing. Negative results in this assay indicate that a sample should be virtually free of mycotoxins. Since the presence of a mycotoxigenic fungus is no assurance that it was producing the mycotoxin, the positive samples left must be analyzed for the presence of mycotoxins using physico-chemical standard methods. Considering consumer protection, false positive results in the PCR are of no concern, but from an economic point of view the number of false positive results should be as low as possible. The usefulness of the PCR methods developed so far to monitor quality and safety in the food an feed industry was already demonstrated. It seems to be even possible by using a quantitative approach to correlate the number of target molecules with fungal biomass or even mycotoxin content. The real time PCR systems have furthermore the potential to be adapted to quantify mRNA, which can be used for monitoring the expression of genes involved in mycotoxin biosynthesis under particular environmental conditions in different foods. Preliminary results on this field are very encouraging.

The most promising breakthroughs in the detection of mycotoxins or mycotoxigenic fungi are expected to be made in the area of sensor technology. Bio-sensors can be used for the detection of very different analytes such as pathogens, pesticides and toxins. They are a subgroup of chemical sensors where the analytical devices are composed of a biological recognition element such as enzymes, antibodies, receptors, proteins, oligonucleotides, or even a whole cell coupled to a chemical or physical transducer, which measures the changes that occur when the sensor couples to its analyte. Only a limited amount of methods are combined and currently exploited for their use in food control. As recognition elements, bioaffinity based receptors that use the selective interaction between ligand and receptor, antibody or nucleic acid are most widely used. As transducers, electrochemical and optical systems have gained practical importance. Microarrays are constructed of a high number of parallel hybrid receptors. They are often referred to as biochips and allow to conduct many analyses in parallel. The advantages over conventional methods of bioanalysis include that a variety of analytes are detected simultaneously in the same sample, that the required sample quantities are minimal and that a high sample throughput is possible. Applications of biochips with low-density arrays, that are arrays with a few hundred different dots, take place in a variety of bioanalytical fields. The systems have high potential for automation and allow the construction of simple and portable equipment for fast analysis.

For the detection of mycotoxins microarrays are under development in which specific antibodies are immobilized on the sensor surface. It is planned to detect at least nine different mycotoxins in parallel (3). Another assay is designed as an inhibition assay (52), in which a fixed concentration of mycotoxin specific antibody is mixed with a sample containing an unknown amount of mycotoxin, whereby antibody and mycotoxin form a complex. The sample is then passed over a sensor surface to which mycotoxin has been immobilized. Non-complexed antibodies are measured as they bind to the mycotoxin on the sensor surface. Mycotoxigenic fungi could also be detected using a microarray with immobilized oligonucleotides, which capture specifically the amplification products generated in a separate mycotoxigenic fungi-specific multiplex PCR or even in an on-chip PCR.

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