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Quantification of the copy number of *nor*-1, a gene of the aflatoxin biosynthetic pathway by real-time PCR, and its correlation to the cfu of *Aspergillus flavus* in foods

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Abstract

A real-time PCR system directed against the *nor*-1 gene of the aflatoxin biosynthetic pathway as a target sequence has been applied to detect an aflatoxinogenic *A. flavus* strain in plant-type foods like maize, pepper and paprika. The system is based on the TaqMan® fluorescent probe technology. The copy numbers of the *nor*-1 gene were compared to conventional cfu data obtained from the same set of samples. In general, a good correlation between *nor*-1 gene copy number and the cfu data was observed; however, the *nor*-1 copy numbers were always higher. It was shown that the system is specific for *nor*-1 containing species.
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Keywords: Real-time PCR; Aflatoxin; *Aspergillus flavus*; *nor*-1; Maize; Pepper; Paprika; Quantification

1. Introduction

Aflatoxins are potent carcinogenic and mutagenic metabolites produced primarily by the fungal species *Aspergillus flavus* and *Aspergillus parasiticus*. These species can contaminate several food commodities including cereals (Pittet, 1988), peanuts (Jelinek et al., 1989) and spices (Bartine and Tantaoui-Elaraki, 1997). Conventional methods to detect, quantify and identify these fungi include cultivation and taxonomic identification at the morphological level. This approach, however, is very time consuming and requires taxonomic skills. Alternative rapid methods like the detection of ergosterol, immunological or impedimetric methods have been described (De Ruiter et al., 1993; Gourama and Bullermann, 1995; Seitz et al., 1977), but they have the drawback of being unspecific. In general, these methods can hardly differentiate between species. The characterization of the aflatoxin biosynthetic genes (Woloshuk and Prieto, 1998), however, made the application of diagnostic PCR methods for the detection of aflatoxinogenic fungi possible (Geisen, 1996; Shapira et al., 1996). The described PCR systems, however, only deliver qualitative results indicating the presence or absence of an aflatoxinogenic fungus. For the estimation of food quality or for the monitoring of the influence of hygienic measures upon the amount of fungi present

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in a food sample, a reliable rapid quantification system is important.

Recently, a technique based on the real-time PCR approach has been developed to detect and quantify fungal conidia in indoor environments (Haugland et al., 1999) or for spores of the pathogenic fungi *Glomus mosseae* and *Phytophthora infestans* (Böhm et al., 1999). Schnerr et al. (2001) described a system directed against the *tri*5 gene to detect and quantify trichothecene producing Fusaria.

The detection principle is based on a PCR reaction with two specific primers which define the target sequence and an additional internal probe which hybridizes between the primers. This additional hybridization step increases the specificity of the reaction and is required for quantification. The internal probe is 5′-labeled with a fluorogenic dye (FAM) and at the 3′-end is ligated to a quencher (TAM). The quencher reduces the fluorescence of the dye as long as it is in close proximity. During the PCR reaction the hybridized probe is degraded by the 5′ 3′-exonuclease activity of the Taq polymerase and the fluorescent dye is released. This process increases the fluorescence, which can quantitatively be determined.

The aim of this work was the establishment of a reliable real-time PCR system for quantification of the *nor*-1 gene and the determination of its correlation to conventional cfu data in various foods.

2. Materials and methods

2.1. Strains and culture collection

Strains used in this work were taken from the culture collection of the Federal Research Centre for Nutrition (Karlsruhe, Germany). The strains were routinely grown in malt extract broth (1.05397, Merck, Damstadt, Germany) at 25 °C under shaking conditions (180 rpm) or on malt extract agar plates (1.05398, Merck, Darmstadt, Germany) at the same temperature.

2.2. Determination of aflatoxin production

For the determination of aflatoxin production the strains were grown at 30 °C on malt extract agar plates or in broth for 3 to 5 days. One colony of each strain or the appropriate amount of the fungal biomass was transferred into a microreaction tube and 500 μl of chloroform was added. The fungal mycelium was extracted for 20 min at room temperature, the mycelium retarded and the chloroform extract was evaporated to dryness in a speed vac concentrator. The residue was redissolved in 10 μl of chloroform and applied onto a TLC plate (Silica gel 60, 1.05748, Merck, Darmstadt, Germany).

As mobile phase toluol (50 volumes), ethylacetate (30 volumes) and acetic acid (4 volumes) were used. Pure aflatoxin (11391, Serva, Heidelberg, Germany) was used as a standard. The spots were visualized under UV light (366 nm).

2.3. Isolation of fungal DNA

The isolation of DNA from pure fungal strains was performed according to a modified method originally described by Yelton et al. (1984). For this purpose, the strains were grown for 72–96 h under shaking conditions (180 rpm) in malt extract broth. After that time the mycelium was harvested by filtration, transferred to a mortar, frozen in liquid nitrogen and ground. The powder was resuspended in lysis buffer (50 mM EDTA, 0.2% SDS, pH 8.5) and immediately heated to 68 °C for 15 min. After a centrifugation for 15 min at 15,000 × *g*, a volume of 7 ml of the supernatant was transferred to a new centrifuge tube and 1 ml of sodium acetate (4 M) was added. This solution was placed on ice for 1 h and centrifuged for 15 min at 15,000 × *g*. After centrifugation the supernatant was transferred into a fresh tube. The DNA solution was phenol extracted by suspending the aqueous phase in the same volume of phenol (saturated with 10 mM Tris/HCl, pH 7.5). Both phases were mixed thoroughly and centrifuged. The upper aqueous phase was transferred into a new microreaction tube. This extraction was repeated three times. After this step, the aqueous phase was extracted with a mixture of chloroform/phenol (1:1), transferred to a fresh tube and extracted with chloroform. The DNA in the aqueous phase was precipitated by the addition of 2.5 volumes of ethanol and redissolved in 500 μl double-distilled water. The purified DNA was either used directly or stored at −20 °C.

2.4. Contamination studies

Powdered pepper, paprika or ground maize kernels were infected with spores of the aflatoxinogenic strain *A. flavus* BFE96 at a concentration of 10^3 spores per gram. The samples were incubated at 30 °C for up to 14 days, depending on the product analysed. The incubation was carried out in a closed chamber where the humidity was kept constant by means of a water reservoir. Each day the samples were thoroughly mixed to ensure homogenous growth of the fungus. At certain time intervals the samples were withdrawn and divided into two parts. One part was used for the determination of cfu and the other part for the isolation of DNA for the real-time PCR reaction.

To analyse the recovery rate of the DNA extraction procedure, pepper was inoculated with known concentrations of spores of *A. flavus* BFE96. For this purpose, a stock solution of freshly prepared spores in 0.85% NaCl was prepared and the spore concentration was determined with a Bürker chamber. The solution was serially diluted in 10-fold steps and used for inoculation of pepper. From these inoculated samples DNA was isolated without further incubation and subjected to real-time PCR.

2.5. Determination of cfu

A sample of 0.25 g was thoroughly mixed with 2.25 ml 0.85% NaCl solution. From this suspension 10-fold dilutions were prepared in 0.85% NaCl solution. A volume of 100 μl of these solutions was plated out on malt extract glucose chloramphenicol plates (malt extract agar 17 g/l, glucose 5 g/l, chloramphenicol (C 0378, Sigma, St. Louis, USA) 100 mg/l) These plates were incubated at 30 °C for 5 days and the colonies were counted. As the natural contamination of the analysed samples by fungi was low, it was easy to identify and count the characteristic *A. flavus* colonies.

2.6. Isolation of DNA from food samples

For the isolation of DNA from infected food samples, 0.1 g of the infected material was transferred to a mortar, frozen in liquid nitrogen and ground. The whole material was used for DNA isolation with the DNeasy Plant Mini Kit (69106, Quiagen, Hilden, Germany). The spin column procedure was carried out essentially as described by the manufacturer. After the last washing step the DNA was eluted from the spin column with 100 μl of double-distilled H_2O. This original DNA was usually further diluted 20-fold to minimize the influence of possibly co-isolated inhibitory substances on PCR. One microliter of that DNA solution was used for the real-time PCR experiments.

2.7. Real-time PCR

The real-time PCR reactions were performed in a GeneAmp 5700® Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA). The primers and the internal probe used in the reaction were suggested by the Primer Express 1.0 software (PE Applied Biosystems). The used primer/probe set had the following nucleotide sequence: nortaq-1, 5′-GTCCAAGCAACAGGCCAAGT-3′; nortaq-2, 5′-TCGTGCATGTTGGTGATGGT-3′; norprobe, 5′-TGTCTTGATCGGCGCCCG-3′ enclosing an amplicon of 66 bp from nucleotide 782 to 847 according the published sequence of the *nor*-1 gene (Trail et al., 1994). For PCR, the TaqMan reagent kit (TaqMan PCR core kit, N808-0228, PE Applied Biosystems) was used according to the suggestions of the manufacturer. For each reaction, 1 μl of the DNA sample solution was mixed with 50 μl of PCR stock solution containing 5 μl of 10 × TaqMan™ buffer, 7 μl of 25 mM $MgCl_2$, 1 μl of each dNTP mixture (10 mM dATP, dCTP, dGTP and 20 mM dUTP), 0.5 μl of the primers (nortaq-1 (25 μM), nortaq-2 (25 μM)) each, 1 μl probe (0.5 nM), 0.5 μl uracil-*N*-glycosylase (1 U/μl), 0.19 μl AmpliTaq Gold (5 U/μl) and 30.3 μl sterile deionized water. The PCR was performed in MicroAmp reaction tubes placed in the 96-well rack of the GeneAmp 5700® Sequence Detection System. After a 2-min incubation at 50 °C to allow for uracil-*N*-glycosylase cleavage, AmpliTaq Gold was activated by incubating for 10 min at 95 °C. Each of the 35 PCR cycles was performed according to the following temperature regime: 95 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s.

A larger PCR fragment of the *nor*-1 gene, generated with the primer nor1 and nor2 (Geisen, 1996), was used to generate the standard curve. The concentration of this standard PCR product was determined in a fluorometer (DyNa Quant 200, Pharmacia, Uppsala,

Sweden) and the number of copies was calculated. These stock solutions were diluted serially by a factor of 10 and the aliquots of the dilutions were used in standard reactions during each setup of the real-time PCR reaction. The concentration of unknown samples was calculated by the GeneAmp 5700® system according the generated standard curve.

2.8. Analysis of the specificity of the reaction

To analyse the specificity of the system, real-time RCR reactions were performed with isolated DNA from pure cultures of different food related fungi. The reactions had the same composition as described above, except that 1 μl of the purified fungal DNA (2 μg/ml) was added instead of the sample DNA.

3. Results

3.1. Primer/probe system for the nor-1 real-time PCR

A conventional non-quantitative PCR reaction with the *nor*-1 gene as the target sequence has been

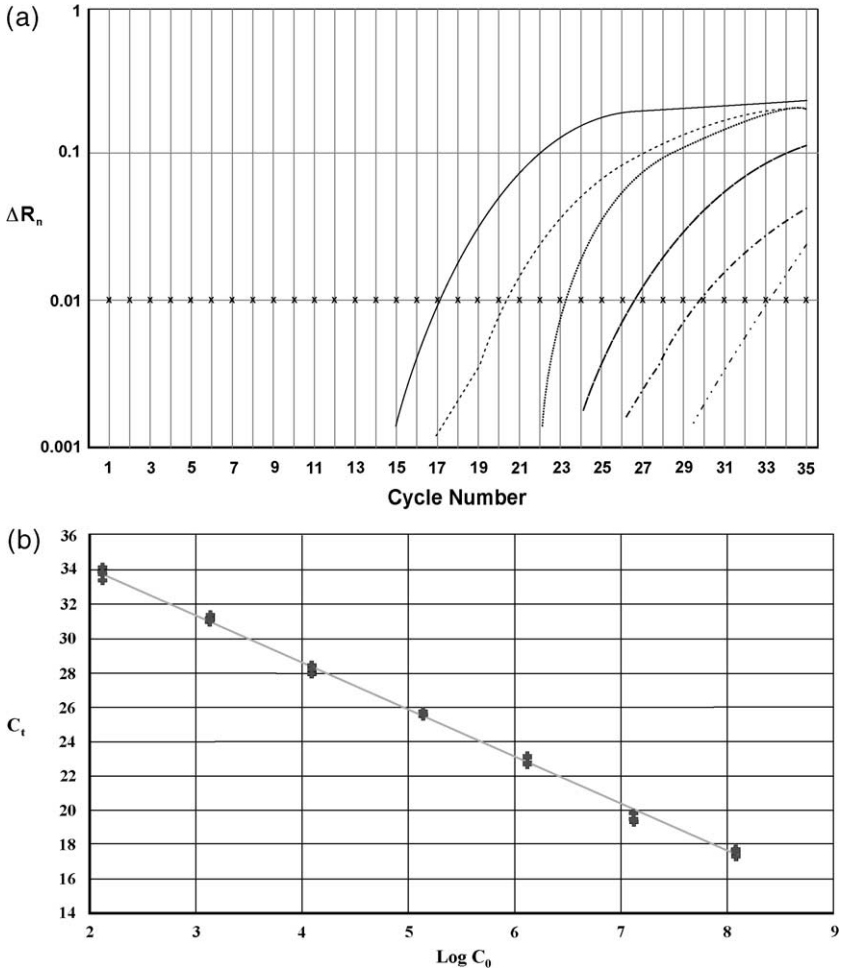

Fig. 1. (a) Amplification plot of a real-time PCR reaction targetted against the *nor*-1 gene with different dilutions of the 400-bp *nor*-1 fragment (—— 10^7, - - - 10^6, · · · · · · · · · 10^5, – – – 10^4, – - – - 10^3, – - - – 10^2). The fluorescence emission (ΔR_n) is plotted against the number of PCR cycles. The threshold value was set at 0.01. (b) Standard curve generated with four independent reactions showing the correlation between cycle number (C_t) and initial copy number of *nor*-1 (log C_0). Slope: -2.77, intercept: 39.77, correlation: -0.995.

Table 1
Specificity of the reaction demonstrated with various food relevant fungi

Strain	Real-time PCR reaction with the *nor*-1 primer/probe
Aspergillus flavus BFE96	+[a]
A. parasiticus BFE293	+
Aspergillus oryzea BFE416	+
Penicillium nalgiovense BFE411	–
Penicillium verrucosum	–
Fusarium moniliforme BFE520	–
F. proliferatum BFE347	–
Fusarium avenaceum BFE569	–
Fusarium sambucinum BFE570	–
Mucor plumbeus BFE365	–
Paecilomyces variotii BFE366	–
Cladosporium cladosporoides BFE370	–
Alternaria alternata BFE376	–

[a] + A *nor*-1 copy number could be determined with the appropriate DNA as template.

described (Geisen, 1996). The *nor*-1 gene codes for the norsolorinic acid reductase, one of the first genes in the aflatoxin biosynthetic pathway (Woloshuk and Prieto, 1998). In the conventional system, the primers nor1 and nor2 have been used and a fragment of 400 bp was produced. For the real-time PCR system, however, the primers have to fulfill specific requirements. First of all, the amplicon length should be in the range of 50 to 100 bp with an appropriate sequence for the hybridization of the internal probe. For this reason, a particular sequence within the 400-bp fragment has been identified by using the Primer Express® Software which fits as good as possible with the requirements for the development of a primer/probe system for real-time PCR. The identified primers/probe were called nortaq-1, nortaq-2 and norprobe. The 400-bp fragment generated with the primers nor1 and nor2 (Geisen, 1996) was used in the reaction as a standard for the copy number.

To demonstrate the functionality of the developed primer/probe system, a real-time reaction was performed with the 400-bp *nor*-1 PCR product which served as the copy number standard. An initial concentration of 10^7 copies of the 400-bp *nor*-1 product was serially diluted by a factor of 10. An example of the reaction curves obtained with this template concentrations is shown in Fig. 1a. They demonstrate that there is a clear correlation between target DNA concentration and changes in fluorescence. The distances of the crossing points between the sample curves and the threshold value correlate with the concentration of template copies. The standard curve generated from these data is shown in Fig. 1b. According to these results the developed primer/probe system (nortaq-1, nortaq-2, norprobe) is functional for the *nor*-1 gene.

3.2. Specificity of the system

To demonstrate the specificity of the system, the real-time PCR assay was performed with purified DNA of different food-related fungi (Table 1). As expected the real-time PCR system gave positive results with species known to carry the *nor*-1 gene of the aflatoxin biosynthetic pathway, like *A. flavus, A. parasiticus*, but also with *A. oryzae*. Reactions using genomic DNA from other species resulted in negative results, indicating that the primer system is indeed specific for *nor*-1 containing species.

3.3. Calibration of DNA isolation

To analyse the recovery rate of the DNA isolation procedure, DNA from pepper samples contaminated with different defined numbers of spores of *A. flavus* BFE96 was analysed by real-time PCR. The real-time PCR data were compared to the spore number (Table 2). According to the results there is a nearly 1:1 correlation between the spore number, determined by microscopic counting, and the real-time PCR data in the analysed range of four orders of magnitude.

Table 2
Correlation of the spore number of *A. flavus* BFE96 inoculated in wheat and the determined *nor*-1 copy number

Dilution	Log spore number/g	Log *nor*-1 copy number/g	Recovery rate[a]
10^7	7.58	7.58	100
10^6	6.58	6.47	98.3
10^5	5.58	5.49	98.4
10^4	4.58	5.29	115.5

[a] The percentage of the recovered *nor*-1 copy numbers were calculated to the inoculated spore numbers (set as 100%).

3.4. Quantification of the nor-1 gene copy in natural samples and correlation to cfu

The spices pepper and paprika, as well as maize kernels, were infected with an *A. flavus* strain (BFE96, 10^3 cfu/g). After different times of incubation, samples were withdrawn and analysed for the numbers of *nor*-1 copies present, as well as for the cfu values. The results are shown in Fig. 2a–c. The growth behaviour of *A. flavus* differs in the various food matrices. In the case of maize and pepper *A. flavus* BFE96 started to grow after 3 days of incubation. In contrast, the lag phase in paprika lasted up to 6 days. However, the cfu values in the stationary phase were nearly the same for all cultures ($\sim 10^7$ cfu/g). The determination of cfu of the uninoculated samples revealed no typical colonies of *A. flavus*. In fact, in general the natural contamination by fungi was low and in the case of pepper no viable fungi at all could be determined, suggesting that this sample has been treated by irradiation or steaming.

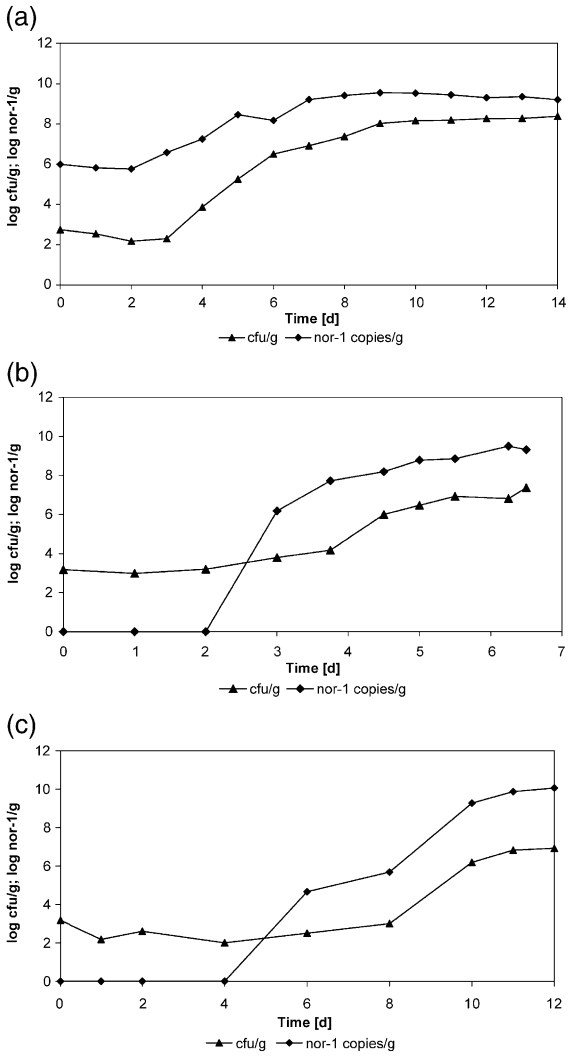

Fig. 2. Comparison of the cfu data and *nor*-1 gene copy numbers of *A. flavus* BFE96 in infected pepper (a), maize (b) and paprika (c) during the incubation period. For each determination several real-time PCR reactions have been carried out. The standard deviation was 0.40 at the beginning and decreased to about 0.26 at the end of the curve for pepper, 0.42 to 0.13 for maize and 0.13 to 0.11 for paprika.

In general, the *nor*-1 gene copies correlate well with the cfu data. However, they were usually higher than the cfu data. In the case of the maize and paprika samples, the real-time PCR approach gave only results after the onset of growth (when the fungal cells started to multiply). In the case of pepper, the real-time PCR approach was able to detect *nor*-1 copies just after inoculation. In fact, a much higher amount of *nor*-1 copies than cfu values could be detected. This number remained constant during the lag phase and increased during the log phase until a maximum was reached.

4. Discussion

The quantification of a fungal contamination is a challenging task because of the nature of the fungal colony, which consists of filamentous mycelial cells and single celled spores. Several methods for the detection or quantification of a fungal contamination have been established, e.g. the determination of the colony forming units (Spangenberg and Ingham, 2000), the measurement of ergosterol content (Seitz et al., 1977), the determination of volatiles (Schnürer et al., 1999) or immunological methods (De Ruiter et al., 1993). Each method uses a specific feature of the fungal colony as the principle for detection and quantification. This implies that the results of the different methods must not directly correlate in a one-to-one ratio.

We describe a real-time PCR method for quantification of the *nor*-1 gene in certain food commodities and we have shown that it correlates with cfu of *A.*

flavus. The real-time PCR data were generally higher than the cfu data. This might be due to several reasons. The determination of cfu mainly reflects the number of spores present in the sample. Mycelial fragments will give rise to only one colony, even if they consist of many cells. In addition, mycelial cells are multinucleate (Gow and Gadd, 1995), meaning that they carry several nuclei. Each nucleus of the target organism carries at least one *nor*-1 gene, all of them contribute to the number of *nor*-1 copies. For this reason mycelial fragments, if present, increase the *nor*-1 copy number at a much higher rate than the cfu number. We have demonstrated by DAPI staining and fluorescent microscopy of cells of *A. flavus* BFE96 that the spores of this strain are uninucleate, whereas the filamentous cells clearly contain several nuclei (in our hands up to 8, data not shown). This is in agreement with literature data, which describe spores of Aspergilli as usually uninucleate, whereas apical cells may contain up to 50 nuclei (Burnett, 1975; Kaminskyj and Hamer, 1998). In addition, dead cells or released DNA can contribute to the number of *nor*-1 copies. In a real-time PCR assay, to quantitatively measure the infection of mice with *A. fumigatus* Bowman et al. (2001) described a similar relationship. They also found a difference between cfu and real-time PCR data. The latter was between 1.2 and 3.5 log units higher, which corresponds to our results.

For the reliability of the method it is essential that the DNA of the contaminating fungus is completely isolated. This was demonstrated by the addition of spores of known concentrations to pepper samples and subsequent real-time PCR. The spore number, as well as the *nor*-1 copy number, was nearly identical over 4 orders of magnitude. Two conclusions can be drawn from these results. First, the isolation of fungal DNA is complete with the method described and, second, there is only one copy of the *nor*-1 gene present in the genome of *A. flavus* BFE96, as we have demonstrated that the spores are uninucleate.

We got no real-time PCR signal just after inoculation with the exception of the pepper sample. The result that the real-time PCR data were higher than the cfu data for the pepper sample, despite the fact that at this stage only uninucleate spores were present, might be due to the possibility that *A. flavus* or *A. parasiticus* DNA was present before inoculation. The fact that we could not detect viable fungi in the pepper sample prior to inoculation suggests that the pepper was treated (e.g. irradiated) and that we measured dead cells.

In the case of paprika and maize no *nor*-1 could be determined during the lag phase of growth. In both cases the first signal could be determined after transition into the log phase. During that phase the *nor*-1 copies increased in parallel with the cfu, albeit at a higher rate. The fact that the real-time PCR approach was not able to detect the *nor*-1 gene during the lag phase, despite the fact that 10^3 cells/g have been inoculated, may be due to several reasons. For the real-time PCR the DNA isolated from 0.1 g sample material was redissolved in 100 μl H_2O (this volume was fixed, because of practical constraints of the method used). One microliter of that solution was used in the PCR reaction. Theoretically, this dilution procedure limits the lower detection level to 10^3 *nor*-1 copies/g. To exclude this possibility we have precipitated the DNA after the isolation procedure and we have used the whole DNA of one extraction in one real-time PCR reaction. The result, however, was the same as with the diluted sample (data not shown), indicating that the dilution is not the reason for the failure of the real-time PCR system to detect *nor*-1 copies in the lag phase. The other possibility is the presence of inhibitors, co-isolated with the DNA, which might inhibit the reaction at low target DNA concentrations. It has been described that several food components are inhibitory to the Taq polymerase (Rossen et al., 1992). It has also been shown that the amount of unspecific DNA present in a sample has an influence on the sensitivity of the system (Färber et al., 1997). In our case, it seems that at low fungal DNA concentrations an inhibition takes place, apparently by competition through high amounts of unspecific plant DNA. With increasing concentrations of specific DNA this effect is obviously minimized. A method to specifically enrich the target sequence compared to the vast amount of plant DNA is currently under development.

The real-time PCR system described is specific for fungal species known to carry the *nor*-1 gene as *A. flavus* and *A. parasiticus*. It also gave positive results with *A. oryzae*. It is known that *A. oryzae* also carries the genes of the aflatoxin biosynthetic pathway (Klich et al., 1995). This situation, however, does not reduce the specificity of the method, as *A. oryzae* is regarded

to be a domesticated form of *A. flavus*, which only rarely occurs in nature (Barbesgaard et al., 1992). It can also be expected that other aflatoxin-producing species like *A. nomius* and *A. pseudotamarii* (Ito et al., 2001) will give positive results with this system. All other tested food-related fungi showed negative results.

Other real-time PCR systems have recently been described for the quantification of the indoor environmental fungus *Stachybotrys chartarum* (Haugland et al., 1999), the two plant pathogens *Phytophthora infestans* and *P. citricola* (Böhm et al., 1999), and the human pathogen *A. fumigatus* (Bowman et al., 2001). Schnerr et al. (2001) described a real-time PCR reaction for trichothecene producing Fusaria, which is directed against the *tri*5 gene. They have used the SYBR green technology, which, however, in our hands had the drawback to be prone to primer dimer formation.

With this work we describe an alternative rapid and reliable method for the quantification of *nor*-1 gene containing strains like *A. flavus* and *A. parasiticus*. The method is functional in various foods and correlates with cfu. The method can be used for quality assessment if one is aware about the general problems of fungal quantification. The method has the potential to be adapted further for the quantification of m-RNA, which can be used for monitoring the expression of aflatoxinogenic genes under particular environmental conditions in different foods. Preliminary results on this field are very encouraging. A non-quantitative method for this aim has already been described (Sweeney et al., 2000).

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