Utilization of the Stratagene Mx3000P QPCR System for the Detection and Quantification of Mycotoxigenic Fungi

Application Note

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Abstract

Rapid and accurate methods are needed to quickly detect mycotoxigenic fungi such as those in the genera of *Aspergillus*, *Penicillium*, and *Fusarium*. This study was conducted to develop and test a multiplex real-time qPCR assay to detect and quantify these agriculturally and economically important fungi. PCR primers were designed to amplify the internal transcribed spacer (ITS) regions of rDNA from three mycotoxigenic genera. Genus-specific probes were designed from the ITS sequences of the most important mycotoxigenic species of *Fusarium*, *Penicillium*, and *Aspergillus*. Fluorophores were attached to the 5′ end of the probes, which were detectable with the Stratagene Mx3000P QPCR system. The linear detection range of the multiplex assay was between 10 pg to 10 ng of DNA. This assay could be used as an initial step to evaluate the potential for mycotoxin accumulation in various agricultural commodities.

Introduction

Mycotoxin detection in stored grain is critical for assuring high quality and food safety. These low molecular weight metabolites, which include aflatoxins, trichothecenes, ochratoxins, and fumonisins, are produced by toxigenic species of the genera *Aspergillus*, *Fusarium*, and *Penicillium*. Consumption of mycotoxin-contaminated food can be detrimental to the health of both humans and livestock.

Knowing the quantity of mycotoxin in a grain source is most important for making a usage decision; however, often information is needed about the potential threat of mycotoxigenic fungi within the grain. Many processes, such as the production of malting barley, place grain under conditions that promote the growth of these mycotoxigenic fungi. Testing grain has conventionally been achieved by microbiological techniques, which require extended incubation time (days), special expertise, and equipment. With large databases containing DNA sequences, PCR technology has made it possible to identify and quantify many important microorganisms by rapid and sensitive assays. The goal of our study was to develop a real-time PCR assay that could detect the three most important genera of mycotoxigenic fungi. This assay is significant because it uses three 5′ fluorophore-labeled hydrolysis probes to produce a multiplex assay that can detect and quantify three mycotoxigenic genera in one reaction tube.

Materials and Methods

DNA, Primers, and Probes

Genomic DNA was isolated from fungal cultures grown on potato dextrose broth and on distiller's grain (DG) at 28°C for 3-7 days by methods previously described (Bluhm et al., 2002; 2004; Suanthie et al., 2009). Purified DNA was quantified by UV spectrometry and serial dilutions were made from an initial concentration of 10 µg/ml. Primer and probe sets were designed with IDT SciTool OligoAnalyzer 3.0 software (Integrated DNA Technologies, Coralville, IA) based on conserved regions in the internal transcribe sequences (ITS) in the ribosomal RNA genes of the mycotoxigenic species of *Aspergillus*, *Fusarium*, and *Penicillium* (Table 1).

Primers/Probes	Sequence (5⟶3′)
ITSPF	**GGC ATC GAT GAA GAA CGC AGC**
ITSPR	**CCT ACC TGA TCC GAG GTC AAC**
FUSP	**TEX-ACG CTC GAA CAG GCA TGC CCG CCA GAA TAC-BHQ2**
ASP1P	**FAM-CAA GCM CGG CTT GTG TGT TGG GTC GYC GTC-TAMRA**
ASP2P	**FAM-AAA GGC AGT GGC GGC ACC ATG TCT GGT-TAMRA**
PENP	**HEX-CTT GTG TGT TGG GCC YCG TCC TCC GA-BHQ1**

[1] Probes were labeled at the 5′ ends with the reporters (TEX, FAM, and HEX) and at the 3′ ends with quenchers (TAMRA, BHQ1, and BHQ2)

Table 1.
Real-time qPCR primers and probes

Materials and Methods (continued)

One primer set (ITSPF and ITSPR) was designed to amplify all the fungal species important in grain production, and genus-specific probes were designed from the sequences between the two primers. Fluorophores FAM, HEX, and Texas Red (TEX) were attached to the 5′ end of the probes, and TAMRA, BHQ1, and BHQ2 served as quenchers at the 3′ end (Table 1).

PCR Assay

A 20 µl reaction was used for amplification. Each reaction contained 10 µl Brilliant II QPCR Master Mix (Agilent Technologies, La Jolla, CA), 1 µl of each primer, 1 µl of each probe, and 3 µl of genomic DNA. The Stratagene Mx3000P QPCR system (Agilent Technologies, La Jolla, CA), equipped with FAM, HEX, ROX, and TET filters, was used for all real-time PCR experiments.

Results and Discussion

Our approach for a multiplex assay starts with primers that amplify genomic DNA from a broad-spectrum fungal species of *Fusarium*, *Aspergillus*, and *Penicillium*. Our analysis of the numerous DNA sequences available indicated that the selected primers ITSPF and ITSPR yielded a PCR product around 300 bp for more than 40 *Aspergillus* species, 23 *Fusarium* species, 32 *Penicillium* species, and 64 genera of other fungi. While the primers assured the amplification of the broadly targeted fungal DNA, genus-specificity was achieved via sequence specific fluorescent probes. Over 1500 sequences from the ITS region of the targeted genera were examined. A single probe (FUSP) was designed to be specific to all *Fusarium* species (Table 1). We were not able to identify useful DNA sequences from *Aspergillus* and *Penicillium* to design a single probe for each genus; therefore, species were prioritized based on their importance to grain production. Two *Aspergillus* probes (ASP1P and ASP2P) were designed that separated the taxonomic subgenera. ASP1P was specific for the important mycotoxigenic species of *Aspergillus*, whereas ASP2P was specific to the xerotolerant *Eurotium* species (subgenus *Aspergillus*, section *Aspergillus*) common in stored grain and dried foods. A BLAST analysis of ASP1P indicated a mismatch of three base pairs with *Eurotium* sequences, and ASP2 has six base pair mismatches with other *Aspergillus* species. Neither probe matched *Aspergillus clavatus*, *A. terreus*, or *A. restrictus* due to the difference in their ITS sequences. The same fluorophore (FAM) was attached to both probes to measure total *Aspergillus* in a sample, and different fluorophores (FAM and HEX) were attached to assess the contribution of each group. The probe designed for *Penicillium* included nine mycotoxigenic species.

Primers and probes were tested on purified DNA from *A. flavus*, *F. graminearum*, and *P. chrysogenum*. Optimum parameters for identification were 10 pmol of each primer and 5 pmol of probe per reaction, with an annealing/extension temperature of 70°C. Detection limits were established for the multiplex assay with serial dilutions of mixed DNA from 10 ng to 1 pg, and standard curves were generated for each fungal species (Figure 1). Resulting Ct (cycle threshold) values correlated with

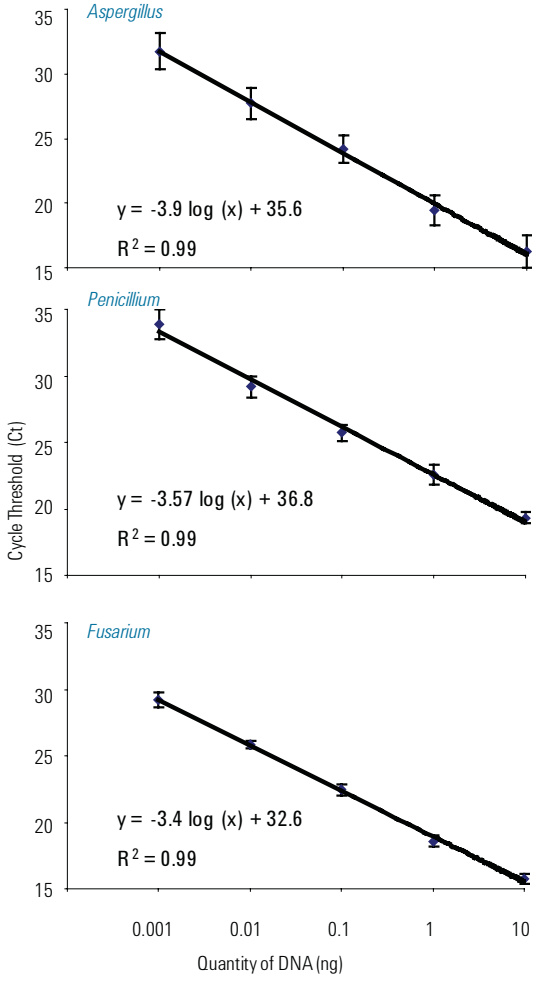

Figure 1.
Standard curves generated for *Aspergillus, Penicillium,* and *Fusarium*.
A mixture of DNA from *A. flavus*, *P. chrysogenum*, and F. *verticilliodes* was serially diluted 10-fold and analyzed by the genus-specific multiplex real-time PCR. The initial DNA concentration was 10 ng/µl for *A. flavus*, *P. chrysogenum*, and *F. verticillioides*. Cycle thresholds (Ct) were plotted against the DNA concentrations. Error bars indicate the standard deviations of Ct between 6 replicates.

the known DNA quantities in the samples, and no interference in either amplification was detected by the presence of 200 ng of non-target DNA. The efficiency values calculated from the slopes of the standard curves indicated that the *Fusarium* and *Penicillium* curves fell in to the desired range (90 to 110 %). The efficiency value for the *Aspergillus* curve (80.5 %) was outside this range, however the values of the standard curves for *Aspergillus* were constant between numerous PCR runs with R2 values of 0.99. We also tested specificity with DNA isolated from 17 fungal isolates. FUSP was specific to the two *Fusarium* species, PENP specific to five *Penicillium* species, and ASP1P and ASP2P specific to three out of the four *Aspergillus* species tested. *A. clavatus* was a non-targeted species.

Application: A Case Study

Distiller's grain (DG) is a by-product of ethanol production from corn. This material is typically dried and used for animal feed. The texture of dried DG is similar to ground coffee. Determining the level of fungal contaminates is difficult because of the enormous amount of yeasts in the product, which was found to be greater that 10^{10} per gram of DG. As a result, detection of storage and mycotoxigenic fungi is an arduous task by traditional plating techniques. We used the multiplex real-time PCR assay developed in this study to analyze a 0.9 metric ton storage sack that was showing signs of mold growth. Six samples were collected from the sack for analysis. All six samples contain predominately *Aspergillus* species varying from 136 to 447 ng DNA per mg of DG (Table 2). A separated PCR with mixed fluorophores on ASP1P and ASP2P indicated that the majority of mold in the samples were *Eurotium* species. Tests for aflatoxin and PCR with primers specific for aflatoxin biosynthetic genes were negative (data not shown). A small amount of *Fusarium* DNA was also detected in one of the samples. Subsequent analysis with PCR primers specific to the trichothecene and fumonisin biosynthetic genes indicated that both types were present.

Sample	Genus-Specific Assay			*Asp/Eur* Assay	
	Aspa	*Fus*	*Pen*	*Asp*	*Eur*
1	228b	0	0	61	163
2	136	0	0	30	141
3	209	0	0	53	123
4	314	0	0	54	244
5	304	32	0	11	300
6	447	0	0	18	383

[a]Asp = *Aspergillus*; Fus = *Fusarium*; Pen = *Penicillium*; Eur = *Eurotium*
[b]ng of DNA per mg of DG; values are the average of 2 replicates.

Table 2.
Analysis of stored distiller's grain (DG) by multiplex real-time qPCR assay.

Conclusion

The multiplex real-time PCR assay developed in this study took advantage of the multiple detection filters in the Stratagene Mx3000P QPCR system. We were able to detect and quantify three genera of mycotoxic fungi. The assay proved useful for the analysis of moldy DG, which has a small particle size and high yeast load that makes this material difficult to analyze by traditional methodology. The multiplex assay is applicable for the analysis of stored grains and foods, and could be used as an initial step of quality assessment in conjunction with other mycotoxin-specific tests such as chemical analysis or standard PCR.

References

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