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Detection of *Aspergillus flavus* in maize kernels by conventional and real-time PCR assays

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Abstract

Aflatoxins are carcinogenic secondary metabolites mainly produced by *Aspergillus flavus* and *A. parasiticus* during infection of susceptible crops, such as maize, groundnut, cotton and chillies. Aflatoxin contamination is considered one of the most serious food safety issues worldwide. To secure the safety of food, regular monitoring of the levels of aflatoxins in foods and feeds is necessary. An alternative could be the detection of the aflatoxin-producing fungi in foods and feeds. In this study a polymerase chain reaction (PCR) method was developed for rapid, specific and sensitive detection of *A. flavus* in maize kernels. *A. flavus*-specific primers based on the O-methyltransferase gene (*omt-A*) that is involved in the aflatoxin B1 biosynthesis, were designed and used to detect the fungus by PCR. The designed primers were highly specific to *A. flavus*. The molecular detection sensitivity of *A. flavus* was 1 ng of purified fungal DNA template in conventional PCR. A real-time PCR assay was standardized for rapid, specific and sensitive detection of *A. flavus* in corn kernels by using these primers. The standard curve obtained showed a linear correlation between copy number of the cloned target DNA sequence of *A. flavus* and Cycle threshold (Ct) values, with R^2 of 0.98. These PCR-based assays may be highly useful in food and feed industries and quarantine laboratories for detection of this fungus.

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Introduction

Aflatoxin contamination in foods and feeds poses a great threat to human and livestock health. These aflatoxins are carcinogenic, secondary metabolites produced by certain strains of *Aspergillus flavus* Link ex Fries and *A. parasiticus* Speare (Bennett and Klich, 2003). Groundnut, maize, cotton and chilli are highly susceptible to invasion by aflatoxigenic *Aspergillus* species and subsequent production of aflatoxins during pre-harvesting, transportation or storage (Moreno and Kang, 1999). Aflatoxins are extremely durable under most conditions of storage, handling and processing of seeds or foods or feeds. It is heat stable and will withstand temperatures up to boiling. Currently, 18 different types of aflatoxins have been identified, with aflatoxin B1, B2, G1, G2 and M1 being the most common. Aflatoxins B1 and B2 are produced by *A. flavus*, whereas B1, B2, G1 and G2 are produced by *A. parasiticus* (Ong, 1975). Aflatoxin M1, a major metabolite of Aflatoxin B1, may be present in milk if obtained from livestock that have consumed aflatoxin B1 contaminated feed (Polan *et al*., 1974). Maize (*Zae mays* L.) is an excellent substrate for the growth of *A. flavus* and aflatoxin production. It has been reported that maize in the field although infected by different species of *Aspergillus*, *A. flavus* is the dominating aflatoxin-producing fungus especially in tropical regions (Calvert *et al.*, 1978, Setamou *et al.*, 1997). Bhat *et al.* (1997) reported that 26% of maize kernels collected from different parts of India were contaminated with AFB1 beyond 30 ppb. Setamou *et al.* (1997) reported that up to 42.5% of maize samples in Benin were contaminated with aflatoxin. Kpodo (1996) reported that the maize samples collected from Ghana contained aflatoxins at levels ranging from 20 to 355 $\mu g kg^{-1}$. Waliyar *et al.* (2003) reported that 43% of maize samples collected from retail shops or super markets in and around Hyderabad, Andhra Pradesh, India were contaminated with toxin with the highest AFB1 level of 806 $\mu g kg^{-1}$. Aflatoxin contaminated diet has been linked with the high incidence of liver cancer (Bababunmi *et al*., 1978). Li *et al*. (2001) reported that the levels of Aflatoxins B1, B2 and G1 were significantly higher in corn from the high incidence areas for human hepatocellular carcinoma. Hence, to secure the safety of food and feed, regular monitoring of aflatoxin levels is necessary. For quantification of aflatoxins in food and feed various analytical techniques such as thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), ultra-performance liquid

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chromatography/tandem mass spectrometry, and enzyme- linked immunosorbent assay (ELISA) have been developed (Trucksess *et al*., 1994; Whitaker *et al*., 1996; Reddy *et al*., 2001; Ventura *et al*., 2006). An alternative could be the detection of the aflatoxin-producing fungi in foods and feeds. The presence of aflatoxigenic fungi in foods and livestock feeds are being monitored in food testing laboratories often by culturing and identification at the morphological level. This approach is time consuming, labour-intensive and requires expertise. In recent years, polymerase chain reaction (PCR)-based methods have emerged as major tools for detection of aflatoxin-producing fungi in foods (Shapira *et al*., 1996; Degola *et al*., 2007; Cruz and Buttner, 2008; Passone *et a*l., 2010; Levin, 2012; Rodriguez *et al*., 2012). In general, these methods are highly sensitive, specific and accurate and can be performed and interpreted by personnel with no specialized taxonomical expertise. Furthermore, these techniques are rapid and less laborious than conventional methods because isolation of the fungus from the infected tissue is not required (Lievens and Thomma, 2005). In the present study we standardized a PCR assay targeting O-methyltransferase gene (*omt-A*) that is involved in the aflatoxin biosynthetic pathway for detection of *A. flavus* in maize kernels. Also we describe here the development of real-time PCR assay that is capable of detecting *A. flavus* in infected maize kernels.

Materials and Methods

Fungal strains

Aspergillus flavus isolates CBE1, CBE2, BSR1, BSR2, BSR3, BSR4, VKR, MDU1, MDU2, SA, ODC, PPT, PLNI, CVP, COT and TJ found in Tamil Nadu, India (Shweta *et al*., 2013) were used in this study. *Aspergillus niger*, *Aspergillus ochraceous*, *Pyricularia grisea*, *Colletotrichum capsici*, *Fusarium verticillioides*, *Fusarium* spp., *Lasiodiploidia theobromae*, *Colletotrichum gloeosporioides*, *Macrophomina phaseolina*, *Chaetomium globosum*, *Rhizoctonia solani*, *Fusarium oxysporum* f.sp. *dianthii*, *Colletotrichum lindemuthianum*, *Colletotricum falcatum*, *Pleurotus sajor-caju*, *Pleurotus eous*, *Trichoderma viride* and *Beauveria bassiana* were obtained from the culture collection of the Department of Plant Pathology, TNAU, Coimbatore, India. Cultures of each isolate were maintained on potato dextrose agar (PDA) (Difco Laboratories, Sparks, MD) under laboratory conditions.

DNA extraction

Fungal isolates were grown in 250 ml conical flasks containing 100 ml potato dextrose broth at room temperature (27 ± 2°C) for 5-7 days. Mycelia were harvested by filtration and freeze-dried. DNA was extracted from freeze-dried mycelia following the method of Liu *et al.* (2000). The genomic DNA was checked by agarose gel electrophoresis and the concentrations of the purified total genomic DNA were determined with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and stored at -20°C for further use.

Primer design

Multiple sequence alignments were performed on sequences of the *omt-A* gene of *A. flavus* (DQ899737, DQ176817, DQ176810, DQ176815 and FN398188) from the National Center for Biotechnology Information GenBank database (*http://www.ncbi.nlm.nih.gov/*) using ClustalW package (Thompson *et al*., 1994). The primers omt-F (5’-GACCAATACGCCCACACAG-3’) and omt-R (5’-CTTTGGTAGCTGTTTCTCGC-3’) designed from the conserved region were commercially synthesized by Operon Company (Operon Biotechnologies, Cologne, Germany) in salt free status.

PCR amplification

PCR reactions were carried out in a total reaction volume of 20 µl, containing 50 ng of template DNA, 10 µl of 2X Go *Taq* master mix (Promega Corporation, Madison, WI) and 10 pmol of each primer. Amplification was performed in a Eppendorf ep gradient S Master cycler (Eppendorf, Hamburg, Germany). The following programme was used to amplify the DNA: 5 min at 94°C (1 cycle); 1 min at 94°C, 1 min at 59°C, and 1 min at 72°C (35 cycles); and 10 min at 72°C. A 10 µl aliquot of PCR products were separated on a 1.2% agarose gel stained with ethidium bromide (0.1 mg/l) and photographed under UV light (302 nm) using an AlphaImager 2000 (Alpha Innotech, San Leandro, CA, USA). A 100- bp ladder (Bangalore Genei Pvt Ltd, Bangalore, India) was used as a size standard.

Sequencing

The amplification product of *A. flavus* MDU2 by using primer pair omt-F and omt-R was sequenced to confirm pathogen identity. DNA was cut out from the agarose gel and purified from the gel slice using QIAquick Gel extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer protocol.

The PCR product was ligated into pGEM-T Easy Vector (Promega, Madison, WI) at 4°C overnight. Ligated DNA was transformed into *Escherichia coli* DH5α (Sambrook and Russell, 2001). Recombinant plasmid DNA was isolated from clones by the use of Wizard Plus Plasmid DNA purification kit (Promega, Madison, WI). The insert DNA was sequenced by using T7 and SP6 vector primers. Sequencing was done at 1st Base Pvt Ltd, Singapore using the Applied Biosystems 3730*xl* DNA analyzer and ABI BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, CA, USA). Database search was performed with the BLAST 2.0 program from the National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD World Wide Web server.

Specificity and sensitivity of the PCR assay

To determine specificity, the primer pair was tested on total genomic DNA (50 ng) from 18 other fungal species as described above. To evaluate the sensitivity of PCR assay, serial dilutions of the total genomic DNA of *A. flavus* MDU2 ranging from 1 pg to 400 ng were prepared and amplified with designed primers as described above. The experiments were repeated at least three times.

Validation of the PCR method for detection of A. flavus on artificially inoculated maize kernels

Maize kernels were surface sterilized with 0.1% $HgCl_2$ solution for 2 min and washed in two changes of sterile distilled water. Seeds were dipped in spore suspension of *A. flavus* strain PPT (1 x 10^6 spores/ml) and placed on sterile petridishes lined with wet blotters. The plates were incubated at 25°C in the dark for 3 days. The inoculated maize kernels were ground with liquid nitrogen and total DNA was extracted by using the CTAB extraction method (Doyle and Doyle, 1987). PCR amplification was performed in 20 µl volumes consisting of 50 ng of template DNA, 10 µl of 2X Go *Taq* master mix (Promega Corporation, Madison, WI) and 10 pmol each of omt-F and omt-R primers. Cycling conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 59°C for 1 min and 72°C for 1 min. After the cycling reactions there was a final extension step of 72°C for 10 min. The amplification products were resolved in 1.2% agarose gel electrophoresis at 80V for 1 h. The gel was treated with ethidium bromide and viewed under UV light.

Real-time PCR assay

Real-time PCR was performed using a Roche LightCycler 96 (Roche Diagnostics GmbH, Manheim, Germany). Each well contains a 20 µl reaction mixture that includes 10 ng of template DNA, 10 pmol each of Omt-F and Omt-R, 10 µl of 2X Light Cycler 480 SYBR Green 1 Master (Roche Diagnostics GmbH, Manheim, Germany). Negative controls containing nuclease-free water instead of DNA were included in every run. Thermal cycling conditions consisted of 7 min at 95°C followed by 35 cycles of 95°C for 30 s 59°C for 30 s and 72°C for 30 s. After the PCR cycle, a melting curve of the product was generated by ramping the temperature to 95°C for 10 s, back to 65°C for 60 s, and then incremental increases of 4.4°C/s up to 95°C with continuous measurement of fluorescence. The threshold cycle (Ct) value for each reaction was assessed using the Light Cycler 96 S W 1.0 software (Roche Diagnostics GmbH, Manheim, Germany). In order to test sensitivity of the primer set and to generate a standard curve of cycle threshold (Ct) versus the log of the target DNA copy number, the recombinant pGEM-T Easy Vector containing the 300 bp target sequence was used. The DNA quantity in the plasmid preparation was determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and copies of target DNA/µl were calculated. A 10-fold dilution series of the target DNA (208 ng/µl; equivalent to 7.3×10^{14} copies/µl) was analyzed to develop amplification plots and standard curve. Negative controls containing nuclease-free water instead of DNA were included in every run. The resulting data were analyzed with the LightCycler Software version 3 to generate standard curve and the standard deviation of each DNA concentration. Following amplification, the PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose gel in Tris-acetate-EDTA (TAE) buffer (0.04M Tris-acetate, 0.001 M EDTA, pH 8.0).

Results and Discussion

In the present study *A. flavus* specific primers were designed based on the *omt-A* gene coding for one of the key enzymes in aflatoxin B1 biosynthesis and used to detect the fungus. DNA extracted from aflatoxigenic and non-aflatoxigenic strains of *A. flavus* was subjected to the PCR using omt-A primers. As expected, a 300-bp DNA fragment was amplified from all 16 isolates of *A. flavus* tested (Figure 1). In order to test the specificity of the primer pairs against *A. flavus*, amplifications were performed using genomic DNA extracted from pure cultures of other fungal pathogens. Assays with *A. flavus* yielded a single amplified DNA fragment of expected size (300 bp) (Figure 2), whereas DNA from the other

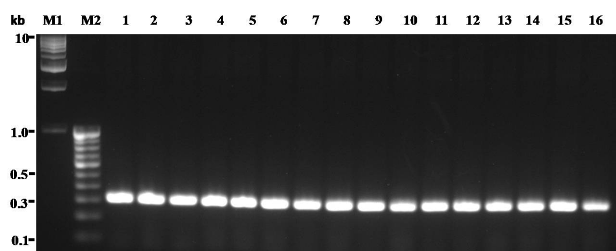

Figure 1. PCR amplification using total DNA extracted from different isolates of *A. flavus* by omt-A primers

Lane M1, 1-kb DNA ladder; lane M2, 100-bp DNA ladder; lane 1, CBE1; lane 2, BSR4; lane 3, BSR3; lane 4, BSR2; lane 5,BSR1;lane6, VKR; lane 7, MDU2; lane 8- SA; lane 9, ODC; lane 10, PPT; lane 11, CBE2; lane 12, PLNI; ; lane 13, MDU1; lane 14,CVP; lane15, COT; lane 16,TJ.

Figure 2. Specificity of PCR assay using omt-A primers and DNA from different fungal pathogens as template

Lane M1, 100-bp DNA ladder; lane 1, *Aspergillus niger*; lane 2, *Aspergillus ochraceous*; lane 3, *Pyricularia oryzae*; lane 4, *Colletotrichum capsici*; lane 5, *Fusarium verticillioides*; lane 6, *Beauveria bassiana*; lane 7, *Fusarium* spp; lane 8, *Lasiodiploidia theobromae*; lane 9, *Colletotrichum gloeosporioides*; lane 10, *Macrophomina phaseolina*; lane 11, *Pleurotus sajor-kaju*; lane 12, *Pleurotus eoeus*; lane 13, *Chaetomium globosum*; lane 14, *Rhizoctonia solani*; lane15, *Trichoderma viride*; lane 16, *Fusarium oxysporum* f.sp. *dianthii*; lane 17, *Colletotrichum lindemuthianum*; lane 18, *Colletotricum falcatum*; lane 19, *Aspergillus flavus*

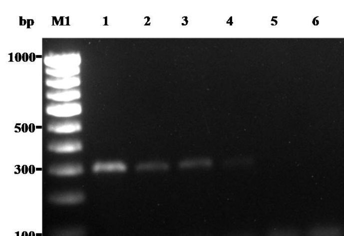

Figure 3. Sensitivity of PCR assay using omt-A primers and different concentrations of *A. flavus* DNA as template

Lane M1, 100-bp DNA ladder; lane 1, 500 ng; lane 2, 100 ng; lane 3, 10 ng; lane 4, 1 ng; lane 5, 0.1 ng; lane 6, 1 pg.

fungal pathogens was not successfully amplified with these primers. To evaluate the sensitivity of PCR assay different dilutions of the *A. flavus* DNA were prepared and amplified with omt-A primers in conventional PCR. The results indicated that the molecular detection sensitivity of *A. flavus* was 1 ng of purified fungal DNA template (Figure 3). The 300 bp amplification product of primer pair omt-F and omt-R from *A. flavus* MDU2 was extracted and sequenced to confirm pathogen identity. The amplicon sequence showed 100% sequence similarity to other *omt-A* gene sequences of *A. flavus* in the GenBank database and it was found reliable enough to use it for detection of *A. flavus*. An experiment was conducted

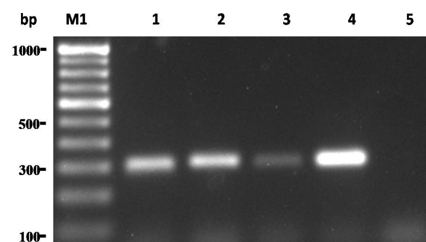

Figure 4. Detection of *A. flavus* in artificially inoculated maize kernels by PCR using omt-A primers

Lane M1, 100-bp DNA ladder; lanes 1-3, artificially inoculated maize kernels; Lane 4, positive control (Pure DNA of *Aspergillus flavus*); Lane 5, healthy maize kernels.

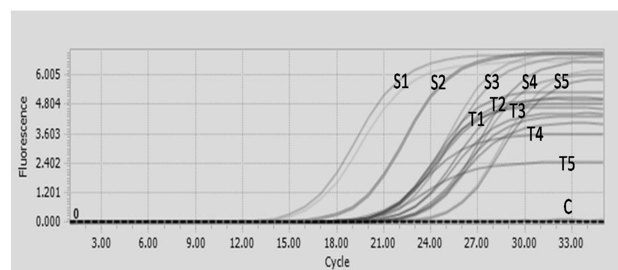

Figure 5. Real-time polymerase chain reaction amplification profile for DNA from *Aspergillus flavus* and DNA extracted from *A. flavus*- infected maize kernels with omt-A primers

For each assay, a series of 10-fold dilutions of cloned target DNA of *A. flavus* (range, 7.3×10^{7} to 7.3×10^{11} copies/μl) were used as the template for real-time PCR (S5–S1, standards dilutions; T1-T5, infected maize samples; C, no template control. Each set of lines represents two replicated amplifications of the same sample. The standard curve had a slope = –2.40 and $R^2 = 0.98$.

to determine whether the developed protocol was effective in the detection of *A. flavus* in maize kernels. The results indicated that the primer pair omt-F and omt-R produced a 300 bp band in all the artificially inoculated maize kernel samples (Figure 4) and no amplification was obtained from DNA extracted from healthy maize kernels.

Several authors have described PCR assays targeting ITS regions or genes involved in aflatoxin biosynthesis for detection of *Aspergillus* species (Shapira *et al.*, 1996, Geisen, 1998; Sweeney *et al.*, 2000; Criseo *et al.*, 2001). Shapira *et al.* (1996) described a PCR assay for detection of aflatoxigenic molds in grains using primers based on genes *ver-1*, *omt-1* and *apa-2*, coding for key enzymes and a regulatory factor in aflatoxin biosynthesis. Geisen (1998) reported a PCR system for detection of aflatoxin producing *Aspergillus* species based on *nor-1*, *ver-1* and *omt-1* genes. Criseo *et al*. (2001) used quadruplex-PCR with four sets of primers for *afl-R*, *nor-1*, *ver-1* and *omt-A* for detection of aflatoxigenic strains of *A. flavus*. Mayer *et al.* (2003) standardized a real-time reverse transcription-PCR system to monitor the production of aflatoxin B1 in wheat by measuring the concentration of nor-1

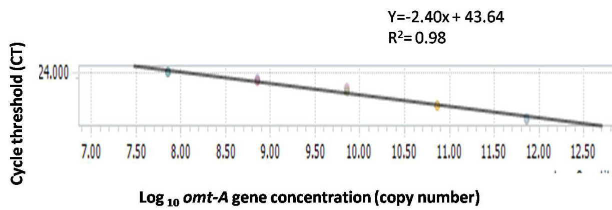

Figure 6. Linear relationship between cycle threshold (CT) and DNA concentrations of *A. flavus* amplified by real-time PCR

CTs were plotted against the log of 10-fold serial dilutions of the 300 bp *omt-A* gene fragment.

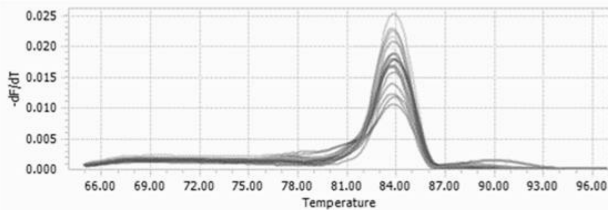

Figure 7. Melting curve for the target amplicon of *A. flavus*, with melting point of 84°C

mRNA. Degola *et al.* (2007) developed a multiplex reverse transcription-polymerase chain reaction (RT-PCR) protocol to discriminate aflatoxin-producing from aflatoxin-nonproducing strains of *A. flavus* and concluded that a good correlation exists between expression of the aflatoxin genes and aflatoxin production.

Classical PCR assays, however, carry several limitations. They are not quantitative and need separation of the products on gels and their visualization under UV light. Real-time PCR assays have been applied in the detection of mycotoxigenic fungi in food products (Leisova *et al*., 2006; Burlakoti *et al*., 2007; Rodríguez *et al*., 2011a; Rodríguez *et al.*, 2011b; Luque *et al*., 2012). Quantification of the DNA template is achieved by continuous monitoring of the amplification by detection of a threshold cycle (Ct) in the early exponential stage of the amplification process, and comparison of amplification curves to known standards. Because of its sensitivity, specificity, and reproducibility, real-time PCR is suitable for detection of trace amounts of fungal DNA (Gayoso *et al.*, 2007). Passone *et al.* (2010) developed a real-time PCR (RT-PCR) system directed against the nor-1 gene of the aflatoxin biosynthetic pathway as target sequence to monitor and quantify *Aspergillus* section Flavi population in peanuts. Cruz and Buttner (2008) developed a primer and probe set targeting the ITS2 region and a small portion of the 28S rRNA gene for rapid detection and quantitation of *A. flavus* using real-time quantitative polymerase chain reaction (QPCR). Rodriguez *et al*. (2012) described real-time PCR (qPCR) protocols based on SYBR Green and TaqMan utilizing primers and probes designed from the o-methyltransferase gene (*omt-1*). The detection limit in all inoculated foods ranged from 1 to 2 log cfu/g for SYBR Green and TaqMan assays. Luque *et al.* (2012) developed a PCR protocol to detect aflatoxigenic molds in food products based on the conserved regions of the O-methyltransferase gene (*omt-1*). The designed primer pair AFF1-AFR3 amplified the expected PCR product (381 bp) in all of the tested aflatoxigenic strains of various species whereas amplification products were not obtained with this primer pair for any of the non-aflatoxigenic reference molds. In the present study a real-time PCR assay specific for *A. flavus* has been developed. In order to establish the procedure, real-time PCR was first performed on purified fungal genomic DNA. Cloned standards of the target DNA sequence from *A. flavus* were developed and analyzed for accurate copy number determinations. The plasmid DNA containing the 300 bp insert was diluted to obtain 10^7 to 10^{11} copies of the target DNA. These target DNA standards were amplified with omt-A primers in real-time PCR to enable estimation of copy numbers of the target DNA in unknown samples. The results showed that Ct values ranged from 15 to 24 for $7.3x10^{11}$ to $7.3x10^7$ copies, respectively, of the target DNA sequence (Figure 5). The samples that showed positive for *A. flavus* infection by conventional PCR assay also showed positive by real-time PCR and Ct values ranged from 20 to 22 for the infected maize kernels. Fluorescence remained below threshold values for water controls and DNA from healthy kernel extract. The standard curves obtained showed a linear correlation between copy number of the target DNA sequence and Ct values, with R^2 value of 0.98 (Figure 6). The melting curve analysis showed a single peak with a melting point of 84°C (Figure 7). Agarose gel electrophoresis of the real-time PCR products showed amplification of the expected size (300 bp) DNA fragment (data not shown).

Conclusions

The present study revealed that the omt-F and omt-R primers could detect both toxigenic and non-toxigenic strains of *Aspergillus flavus* by SYBR Green real-time and conventional PCR in infected maize kernels. The samples that showed positive for *A. flavus* infection by conventional PCR also showed positive by real-time PCR; however, real-time PCR was more sensitive in detecting *A. flavus* in infected kernels. It has been reported that the coding sequence of *omt-A* gene from *A. parasiticus* strain SRRC143 showed more than 97% sequence identity with that from *A. flavus* strain CRA01-2B (Yu *et al*., 1995). Hence the primer set specific for the *omt-A* gene may

also be applied to the screening of food commodities and feeds for the presence of *A. parasiticus*. The RT-PCR assay developed in this study may be highly useful for rapid and accurate detection of this important fungus in foods and feeds and would be of considerable use to seed-testing laboratories and in plant quarantine laboratories. Since the presence of *A. flavus* is of no assurance that it will produce the aflatoxin, the positive samples in PCR must be analyzed for the presence of aflatoxin.

Acknowledgements

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