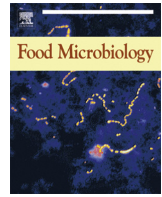
Food Microbiology 44 (2014) 142–148

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm



# Rapid detection of aflatoxin producing fungi in food by real-time quantitative loop-mediated isothermal amplification

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ARTICLE INFO

*Article history:*
Received 4 December 2013
Received in revised form
10 April 2014
Accepted 6 June 2014
Available online 14 June 2014

*Keywords:*
*Aspergillus*
Aflatoxin
Spore
Food
Real-time LAMP

ABSTRACT

Aflatoxins represent a serious risk for human and animal health. They are mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus* but also by *Aspergillus nomius*. Three species specific turbidimeter based real-time LAMP (loop-mediated isothermal amplification) assays were developed to quantify the three species individually in conidial solutions and to define contamination levels in samples of shelled Brazil nuts, maize, and peanuts. Standard curves relating spore numbers to time to threshold ($T_t$) values were set up for each of the species. Assays had detection limits of 10, 100 and 100 conidia per reaction of *A. flavus*, *A. parasiticus*, and *A. nomius*, respectively. Analysis of contaminated sample materials revealed that the *A. nomius* specific real-time LAMP assay detected a minimum of 10 conidia/g in Brazil nuts while assays specific for *A. flavus* and *A. parasiticus* had detection limits of $10^2$ conidia/g and $10^5$ conidia/g, respectively in peanut samples as well as $10^4$ conidia/g and $10^4$ conidia/g, respectively in samples of maize. The real-time LAMP assays developed here appear to be promising tools for the prediction of potential aflatoxigenic risk at an early stage and in all critical control points of the food and feed production chain.

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## 1. Introduction

Aflatoxins are a class of fungal secondary metabolites with high toxicity and carcinogenicity toward animals and humans and are thus a topic of high concern in the food and feed industry. Aflatoxins B1, B2, G1 and G2 are of significance as contaminants of commodities used in the production of foods and feeds. Aflatoxin $B_1$ ($AFB_1$) has been classified as a human carcinogen (group 1A) by the International Agency for Research on Cancer (IARC, 1993). The evidence regarding the potential carcinogenicity of aflatoxins has forced governmental regulatory agencies to establish very low tolerances in food, including peanuts and related products, in order to prevent trade of commodities contaminated by these toxins at levels exceeding a maximum limit (van Egmond and Jonker, 2004). The Food and Agriculture Organization (FAO) estimates that 25% of the world's food crops are affected by aflatoxins, which have significant effects also on livestock and poultry (Adams and Motarjemi, 1999). In addition to health concerns related to aflatoxins, the rejection of contaminated bulk commodities such as peanuts, rice, sorghum and maize as well as products of smaller concern like pistachios, hazelnuts, Brazil nuts and nutmeg with aflatoxin concentrations exceeding the maximum acceptable level results in large economic losses, worldwide.

Aflatoxigenic species occur in sections *Flavi*, *Nidulantes* and *Ochraceorosei* of the genus *Aspergillus* (Varga et al., 2009), with section *Flavi* containing the majority of potential producers (Cary et al., 2005; Pildain et al., 2008). *Aspergillus flavus* and *Aspergillus parasiticus* are the most common filamentous fungi associated with aflatoxin contamination of commercially important agricultural commodities (i.e., groundnut kernels, maize, rice and sorghum grain) (Kumar et al., 2008). In addition, Olsen et al. (2008) suggested that the less regularly occurring *Aspergillus nomius* may be highly important as producer of aflatoxins in Brazil nuts. As a consequence, also this fungus as well as other potential producers of B and G aflatoxins should be carefully examined since the findings may influence strategies for prevention and control of aflatoxins in Brazil nuts. Only recently, Calderari et al. (2013) verified that *A. nomius* and *A. flavus* are the main aflatoxin producers at different stages of the Brazil nut production chain.

The level of fungal infection in food and feed commodities as well as the identification of major species is important since the result can give an indication of the food quality as well as of the potential future risk of mycotoxins being present in a sample (Suanthie et al., 2009). Possible interventions aimed at lowering or preventing aflatoxin contamination in agricultural commodities

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http://dx.doi.org/10.1016/j.fm.2014.06.004
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include good agricultural practice with early harvesting and proper drying of the commodity, biological and chemical control, and breeding for resistance (Turner et al., 2005). All these strategies require effective and rapid control schemes. Rapid and sensitive methods for detection and differentiation of potential aflatoxigenic species in raw commodities and in food and feed products in order to estimate any potential health risk are highly needed (Valasek and Repa, 2005). For the detection of molds, methods such as the traditional mycological methods (Pitt and Hocking, 2009), enzyme-linked immunosorbent assay (Notermans et al., 1986) as well as PCR, qPCR, RT-PCR and multiplex qPCR (Shapira et al., 1996; Sardiñas et al., 2011; Haugland et al., 2002; Rodríguez et al., 2012a; Bernáldez et al., 2014) have been developed and applied. Also DNA-based biosensors have been recently developed for the detection of aflatoxin producing molds (Tombelli et al., 2009). However, the traditional mycological methods being used to assess mold presence in commodities are time-consuming, labor-intensive, require lab facilities and mycological expertise and, above all, do not readily allow for the identification of mycotoxigenic strains. The molecular detection methods named above are costly and require trained personnel, although they have been described as more rapid, sensitive and specific as compared to microbiological methods. As an alternative technology, loop-mediated isothermal amplification (LAMP) of DNA was described as a specific, rapid, cost-effective, and easy-to-use method by Notomi et al. (2000). Only few applications of LAMP for the detection of fungal organisms have been described so far. Most recently, Niessen et al. (2013) reviewed the application of LAMP-based methods for the detection and identification of food borne bacterial pathogens and toxicants as well as mycotoxin producing food-borne fungi.

The aim of the present work was to use three sets of LAMP primers previously introduced by Luo et al. (2012) for the development of turbidimeter based real-time LAMP assays specific for *A. flavus*, *A. parasiticus* and *A. nomius*, respectively, and to apply those new assays for the quantification of these three important aflatoxin-producing species in sample materials.

## 2. Materials and methods

### 2.1. Material

#### 2.1.1. Preparation of spore suspensions

Fungal colonies of *A. flavus* CBS 113.32, *A. parasiticus* CBS 126.62, and *A. nomius* CBS 260.86 were grown on MEA plates (3% (w/v) malt extract, 0.3% (w/v) soy peptone, pH 5.2) at ambient temperature under diffuse daylight until abundant sporulation occurred. Conidial suspensions were prepared using the method described by Luo et al. (2014). Conidia were harvested from plates and spun at 5 000x g for 5 min at ambient temperature. The pellet was washed twice with sterile deionized water and intermediate centrifugation under the conditions used previously. Washed conidia were re-suspended in 2 ml sterile deionized water. Conidial concentrations were assessed by counting of an appropriate dilution in a Thoma type counting chamber (depth 0.1 mm).

#### 2.1.2. Preparation of contaminated sample materials

Shelled Brazil nuts, maize and peanuts with their red seed hulls (teguments) removed were ground in a coffee grinder (MKM 6003, Bosch, Germany) at maximum speed for 3 min. One gram of ground samples were inoculated with 200 µl conidial suspensions of either of the three fungi at concentrations $5 \times 10^6$, $5 \times 10^5$, $5 \times 10^4$, $5 \times 10^3$, $5 \times 10^2$ and $5 \times 10^1$ conidia/ml to result in conidial loads of $10^6$–10 conidia/g, respectively. DNA was extracted from samples immediately after inoculation with conidia in order to prevent germination.

### 2.2. DNA extraction

Highly purified fungal genomic DNA (gDNA) of reference strains *A. flavus* CBS 113.32, *A. parasiticus* CBS 126.62, and *A. nomius* CBS 260.86 was used as positive controls throughout the study. The mycelia were finely ground using the method described by Luo et al. (2012). Ground mycelia were subjected to DNA-extraction according to the method described by Niessen and Vogel (2010). A rapid extraction protocol as described in Luo et al. (2014) was used to prepare gDNA from 10-fold serial dilutions of conidia of the three reference strains involving vortexing for 10 min at maximum speed for cell disruption and boiling for 10 min.

DNA extraction from samples of Brazil nuts, peanuts and maize with and without artificial inoculation with a 10-fold serial dilution of conidia of the three reference species was performed according to the CTAB method described by Alary et al. (2002) with some modifications. 5 ml of CTAB extraction buffer (20 g/l CTAB, 1.4 M NaCl, 100 mM Tris HCl, 20 mM EDTA, pH 8.0) were added to 1 g of finely ground sample in a 50 ml Falcon tube, homogenized by vortexing and treated with ultrasonic for 3 min. A sonotrode S14 connected to a UP200S ultrasonication apparatus (Dr. Hielscher, Berlin, Germany) was used at 50% intensity with maximum amplitude. Following ultrasonication, samples were incubated for 30 min in a water bath at 65 °C. During incubation the tubes were mixed every 5 min by inversion. The solution was centrifuged at 15 000× *g* for 15 min at 20 °C to pellet the solid debris followed by transfer of the supernatant to a new sterile 15 ml Falcon tube before adding an equal volume of chloroform-isoamyl alcohol (24:1). The mixture was homogenized by vortexing for 30 s and phases were separated by centrifugation at 12 000× *g* for 15 min at 20 °C. The upper aqueous phase was transferred to a new 15 ml Falcon tube and 2 volumes of CTAB precipitation buffer (5 g/l CTAB, 40 mM NaCl, pH 8.0) were added. The mixture was homogenized and incubated at room temperature for 1 h before centrifugation at 12 000× *g* for 15 min at 20 °C. The supernatant was discarded and the pellet was dissolved in 1 ml of 1.2 M NaCl. One milliliter of chloroform-isoamyl alcohol (24:1) was added and the mixture was homogenized by vortexing for 30 s before centrifugation at 12 000× *g* for 10 min at 20 °C. The upper phase was transferred to a new 1.5 ml reaction tube, 0.6 vol of isopropanol at ambient temperature was added and the mixture was mixed by thoroughly inverting before centrifugation at 17 000× *g* for 15 min at 20 °C. The DNA pellet was washed with 700 µl of 70% ethanol at −20 °C, and centrifuged again and washing was repeated with 500 µl of ice cold 70% ethanol. The DNA pellet was dried under a fume cabinet and re-dissolved in 30 µl of sterile deionized water.

### 2.3. Real-time LAMP reaction

The *A. flavus* ID58, *A. parasiticus* ID153 and *A. nomius* ID9 LMAP assays as described in Luo et al. (2012) were used for real-time LAMP with minor modifications. The LAMP reaction was carried out in a total 25 µL reaction volume using the mixture containing per reaction: 2.5 µl 10 × LAMP buffer (200 mM MOPS, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, pH 8.8) (all chemicals from Sigma–Aldrich, Taufkirchen, Germany), 3.5 µl dNTP mix (10 mM each dGTP, dATP, dTTP, dCTP, Fermentas, St. Leon-Rot, Germany), 1 µl 200 mM $MgCl_2$, 2.6 µl primer mix (1.6 µM FIP, 1.6 µM BIP, 0.8 µM Loop F, 0.8 µM Loop B, 0.2 µM F3 and 0.2 µM B3, see Luo et al. (2012) for primer sequences), 1.0 µl (8 U) *Bst* 2.0 DNA polymerase, large fragment (8000 U/ml, New England Biolabs, Frankfurt, Germany). Three sets of LAMP primers targeting the *A. flavus acl*-1 gene, the *A. parasiticus amy*-1 gene, and the *A. nomius amy*-1 gene as described previously (Luo et al., 2012) were used. For the assays, 5.5% (v/v), 3.7%, 5.5% formamide was added, respectively. Sterile deionized water was added to result in a 25 µl total reaction volume including 5 µl DNA

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