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LAMP-based group specific detection of aflatoxin producers within *Aspergillus* section *Flavi* in food raw materials, spices, and dried fruit using neutral red for visible-light signal detection

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ABSTRACT

Aflatoxins can be produced by 21 species within sections *Flavi* (16 species), *Ochraceorosei* (2), and *Nidulantes* (3) of the fungal genus *Aspergillus*. They pose risks to human and animal health due to high toxicity and carcinogenicity. Detecting aflatoxin producers can help to assess toxicological risks associated with contaminated commodities. Species specific molecular assays (PCR and LAMP) are available for detection of major producers, but fail to detect species of minor importance. To enable rapid and sensitive detection of several aflatoxin producing species in a single analysis, a *nor1* gene-specific LAMP assay was developed. Specificity testing showed that among 128 fungal species from 28 genera, 15 aflatoxigenic species in section *Flavi* were detected, including synonyms of *A. flavus* and *A. parasiticus*. No cross reactions were found with other tested species. The detection limit of the assay was 9.03 pg of *A. parasiticus* genomic DNA per reaction. Visual detection of positive LAMP reactions under daylight conditions was facilitated using neutral red to allow unambiguous distinction between positive and negative assay results. Application of the assay to the detection of *A. parasiticus* conidia revealed a detection limit of 211 conidia per reaction after minimal sample preparation. The usefulness of the assay was demonstrated in the analysis of aflatoxinogenic species in samples of rice, nuts, raisins, dried figs, as well as powdered spices. Comparison of LAMP results with presence/absence of aflatoxins and aflatoxin producing fungi in 50 rice samples showed good correlation between these parameters. Our study suggests that the developed LAMP assay is a rapid, sensitive and user-friendly tool for surveillance and quality control in our food industry.

1. Introduction

Aflatoxins are a group of mycotoxins exhibiting acute immuno- and hepato-toxicity in humans and animals (Cullen and Newberne, 2013). In addition, it has a very high carcinogenic potential following chronic exposure (Eaton and Gallagher, 1994). Due to their pronounced health effects, aflatoxins are an imminent threat for the safety of food and feed, worldwide (Milićević et al., 2010). Many countries in the world, including the United States and the European Community, have established strict thresholds for aflatoxins in certain food commodities (Egmond et al., 2007). The latest outbreak of acute human aflatoxicosis was identified among patients living in Kenya's eastern and central provinces, where 125 persons died as a result of aflatoxin poisoning from ingestion of contaminated commercial maize products in April 2004 (Gieseker et al., 2005; Probst et al., 2007). In addition to maize, aflatoxigenic fungi can contaminate several other food commodities including cereals (Mitchell et al., 2016) dried fruits, nuts, and edible seeds (Masood et al., 2015), rice (Reddy et al., 2009b), spices and herbs (Kabak and Dobson, 2017; Tripathy et al., 2015), figs (Doster et al., 1996), green coffee beans (Nakajima et al., 1997), cocoa and its products (Turcotte et al., 2013) and Brazil nuts (Freitas-Silva and Venâncio, 2011). Food and feed raw materials are especially susceptible to invasion by aflatoxigenic *Aspergillus* spp. under warm and dry climatic conditions. Aflatoxins can be produced at all stages of commodity processing including growth in the field, harvest, processing, transportation and storage (Cotty and Jaime-Garcia, 2007; Ellis et al., 1991). Aflatoxigenic species occur in three sections of the fungal genus *Aspergillus*, i.e. sections *Flavi*, *Nidulantes*, and *Ochraceorosei* (Varga et al.,

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2011). Among these, section *Flavi* currently contains 16 species capable of producing a wide array of toxic compounds (Varga et al., 2015), the most common of which are aflatoxins type B and type G, cyclopiazonic acid (CPA), aspergillic acid and kojic acid (Ito et al., 2001; Samson et al., 2010). Regarding the economic significance of aflatoxin producers, *A. flavus* and *A. parasiticus* are the most important species (Bennett et al., 1988). *A. oryzae* and *A. sojae*, which are used as non-aflatoxinogenic Koji starters during the production of Shoyu (Japanese fermented Soy sauce) and other traditional fermented food, have been found to be synonyms for *A. flavus* and *A. parasiticus*, respectively (Kurtzman et al., 1986), although some toxin producing strains were detected (Jørgensen, 2007). In addition, *A. nomius* and *A. pseudonomius* were found to be highly important as producers of aflatoxins in Brazil nuts (Massi et al., 2014; Olsen et al., 2008) and also in cocoa (Copetti et al., 2011). Apart from the established set of three major aflatoxigenic species, a variety of taxa within *Aspergillus* section *Flavi* have been recently described as producers of aflatoxins, including *A. bombycis* (Peterson et al., 2001), *A. arachidicola* and *A. minisclerotigenes* (Pildain et al., 2008), *A. novoparasiticus* (Gonçalves et al., 2012), *A. parvisclerotigenus* (Frisvad et al., 2005), *A. pseudocaelatus* and *A. pseudonomius* (Varga et al., 2011), *A. pseudotamarii* (Ito et al., 2001), *A. mottae*, *A. sergii*, and *A. transmontanensis* (Soares et al., 2012), *A. novoparasiticus* (Gonçalves et al., 2012) and *A. togoensis* (Samson and Seifert, 1986). Moreover, *A. ochraceoroseus* (Bartoli and Maggi, 1978) and *A. rambelii* (Frisvad et al., 2005) are aflatoxinogenic species in section *Ochraceorosei*, and in section *Nidulantes*, *Emericella astellata* (Frisvad et al., 2004), *E. venezuelensis* (Frisvad and Samson, 2004), and *E. olivicola* (Zalar et al., 2008) have been identified as producers of aflatoxins and other potent mycotoxins. The presence of any of the above mentioned species in food or in commodities used for food production may be indicative for the presence of aflatoxins. Therefore, knowledge about the level of aflatoxinogenic molds is important as an indicator for the quality of commodities as well as for aflatoxins being potentially produced later during storage and processing (Shapira et al., 1996). Classical methods of identification and detection of aflatoxin producing fungi in food and food raw materials include cultivation on different agar media as well as micro- and macro- morphological analysis (Klich, 2002). Differential growth media such as Aspergillus differential medium (ADM, (Bothast and Fennell, 1974), coconut cream agar (CCA (Dyer and McCammon, 1994) or Aspergillus flavus-parasiticus agar (AFPA (Pitt et al., 1983) have widely improved microbiological analyses in the past. However, these approaches are only fit to the analysis of the most common aflatoxin producing species. Moreover, they are time-consuming, labor- and cost-intensive and require considerable mycological expertise and dedicated lab facilities. Moreover, methods involving cultivation lack enough sensitivity since, on selective and semi-selective media, only a small proportion of the fungal propagules present in a sample will actually survive and form countable colonies (Beuchat, 1993). As an alternative, molecular methods such as PCR and real-time PCR have been applied to the detection of *Aspergillus* species by amplifying housekeeping genes such as *btub* (Rodríguez et al., 2012; Sweeney et al., 2000), genes coding for ribosomal RNA (Bu et al., 2005; Cruz and Buttner, 2008; Sardiñas et al., 2011) or, most commonly, genes involved in the biosynthesis of aflatoxins (Ahmad et al., 2014; Färber et al., 1997; Geisen, 1996; Mangal et al., 2016; Manonmani et al., 2005; Mayer et al., 2003; Mideros et al., 2009; Passone et al., 2010; Rahimi et al., 2008; Rodríguez et al., 2012; Shapira et al., 1996; Sweeney et al., 2000). However, the requirement for trained personnel and special equipment and reagents hamper the broad practical application of PCR-based methods in low-tech environments or in the field. Another obstacle is the fact that PCR-based methods regularly need some kind of DNA clean up and concentration prior to analysis. Moreover, detection of contaminants directly from infected commodities by PCR is mostly hampered by the complex microbiota often containing closely related species and a complex mixture of compounds that may affect the efficiency and sensitivity of a PCR assay (Rossen et al., 1992; Schrader et al., 2012).

As an alternative to PCR-based analysis, loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000) has been described as an easy and rapid diagnostic tool. LAMP makes use of four primers with six different binding sites in the target DNA which are required to bind properly in order to initiate DNA amplification by a thermophilic DNA polymerase with high strand displacement activity (Notomi et al., 2000). The high number of binding sites enables highly specific amplification of target DNA. Due to their high fidelity, *Bst*-type DNA polymerases enable biosynthesis of detectable amounts of amplicon DNA within 60 to 90 min. The application of additional loop primers further enhances reaction speed so that a result can be seen even within 30 to 60 min (Nagamine et al., 2002). In addition to high specificity and sensitivity of LAMP assays, they are relatively unaffected by inhibitors from growth media or from the food matrix analyzed (Kaneko et al., 2007), and can therefore be performed with a minimum of lab equipment. LAMP assays have been described for the detection of bacteria (Niessen et al., 2013), viruses (Parida et al., 2008) and fungi (Niessen, 2015), but also for a variety of protists, animals and plants. Even though LAMP assays for the specific detection of particular aflatoxigenic *Aspergillus* spp. have already been developed and applied (Luo et al., 2014a; Luo et al., 2012; Luo et al., 2014b), no such assay is available for the rapid and sensitive detection of a broad spectrum of different aflatoxin producing species in a wide range of different food commodities.

LAMP signal detection can be attained directly by agarose gel electrophoresis and ethidium bromide staining of the typical ladder-like pattern of DNA concatemers, resulting from DNA amplification during LAMP (Gill and Ghaemi, 2008; Notomi et al., 2000). For indirect signal detection, methods can be used which either apply dedicated equipment, such as a real-time fluorimeter or real-time turbidimeter, or use naked eye-based detection of fluorescent intercalating dyes (e.g. ethidium bromide, SYBR green 1), or the complexometric indicator calcein under a UV lamp. Recently, visible light signal detection of LAMP reactions has been achieved using hydroxy naphtol blue (HNB, Goto et al., 2009) which follows a similar mechanism as calcein. As an alternative, Tanner et al. (2015) suggested the use of pH-sensitive dyes in a weakly buffered LAMP master mix that change color upon acidification of positive LAMP reactions during DNA biosynthesis.

The aim of the current study was to develop and evaluate a simple and rapid LAMP-based assay for group-specific detection of aflatoxin producing species within *Aspergillus* section *Flavi*, and to apply this assay for the detection of target fungi from purified DNA, spore suspensions, artificially contaminated samples, and naturally contaminated rice, maize, nuts, spices and dried fruits. Moreover, this study was aimed at assessing the usefulness of neutral red as an indicator for visible LAMP signal detection under daylight conditions.

2. Materials and methods

2.1. Fungal cultures and culture conditions

A complete list of fungal isolates used during this study is shown in Table S1 in the Supporting materials. All media were sterilized by autoclaving at 121 °C for 20 min prior to use. For long term preservation, fungal cultures were grown on malt extract broth without shaking (ME broth, per liter 20 g malt extract (Applichem, Darmstadt, Germany), 2 g soy peptone (Oxoid, Basingstoke, England), pH 5.4) with Leca®-clay granules added as solid support. Granules were transferred to 80% (v/v) glycerol and maintained at − 80 °C. Working cultures of *Fusarium* spp. were grown on synthetic nutrient agar (SNA, Nirenberg, 1976). All other genera were cultivated on malt extract agar plates (MEA, per liter 20 g malt extract, 2 g soy peptone, 15 g agar (Difco, Heidelberg, Germany), pH 5.4). All cultures were grown and maintained at ambient temperature (AT, 22 ± 1 °C). MEA was also used for morphological investigation and preparation of conidial suspensions from *Aspergillus*

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