LAMP-PCR detection of ochratoxigenic *Aspergillus* species collected from peanut kernel

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ABSTRACT. Over the last decade, ochratoxin A (OTA) has been widely described and is ubiquitous in several agricultural products. Ochratoxins represent the second-most important mycotoxin group after aflatoxins. A total of 34 samples were surveyed from 3 locations, including Mecca, Madina, and Riyadh, Saudi Arabia, during 2012. Fungal contamination frequency was determined for surface-sterilized peanut seeds, which were seeded onto malt extract agar media. *Aspergillus niger* (35%), *Aspergillus ochraceus* (30%), and *Aspergillus carbonarius* (25%) were the most frequently observed *Aspergillius* species, while *Aspergillus flavus* and *Aspergillus phoenicis* isolates were only infrequently recovered and in small numbers (10%). OTA production was evaluated on yeast extract sucrose medium, which revealed that 57% of the isolates were *A. niger* and 60% of *A. carbonarius* isolates were OTA producers; 100% belonged to *A. ochraceus*. Only one isolate, morphologically identified as *A. carbonarius*, and 3 *A. niger* isolates unstably produced OTA. A polymerase chain reaction (PCR)-based identification and detection assay was used to identify *A. ochraceus* isolates. Using the primer sets OCRA1/OCRA2, 400-base pair PCR fragments were produced only when genomic DNA from *A. ochraceus* isolates was used. Recently, the loop-mediated isothermal amplification assay using recombinase polymerase amplification chemistry was used for *A. carbonarius*

and *A. niger* DNA identification. As a non-gel-based technique, the amplification product was directly visualized in the reaction tube after adding calcein for naked-eye examination.

INTRODUCTION

Black aspergilla, including *Aspergillus carbonarius*, *Aspergillus awamori*, and *Aspergillus niger*, have been reported to be the main producers of ochratoxin A (OTA) (Abarca et al., 2004). *Aspergillus ochraceus* can infest many agricultural commodities, including cereals, coffee, peanuts, grapes, and others, often producing numerous mycotoxins such as OTA, viomellein, and penicillic acid. *A. carbonarius* is considered to be responsible for OTA contamination in grapes, wines, and coffee (Battilani et al., 2006). Other nonochratoxigenic black aspergilli collected from grapes in European vineyards are typically found to belong to the species *Aspergillus tubingensis* and *Aspergillus uvarum* (Perrone et al., 2007; Varga et al., 2011). OTA is a nephropatic, teratogenic, and carcinogenic mycotoxin found in food commodities and produced by some food-borne *Aspergillus* species (Belli et al., 2004). The *Aspergillus* species section Nigri are difficult to identify and classify; however, some taxonomic systems have been suggested. *Aspergillius* species identification based on cultural, morphological, and biochemical characters is laborious and not always straight forward. Molecular markers are useful for these studies (Geiser et al., 2007). Molecular methods have decreased the number of synonym names of defined species within the black aspergilli and supported the division of this "aggregate" in 2-4 morphologically undetectable species: *A. niger*, *A. tubingensis*, *Aspergillus foetidus*, and *Aspergillus brasiliensis* (Abarca et al., 2004). These 4 species are very difficult to distinguish using 23 cultural and morphological criteria, such as conidial shape, color, and size (Samson et al., 2004). Recently, some polymerase chain reaction (PCR)- and real-time PCR-based assays have been established for the fast and specific identification of aspergilla collected from agricultural commodities (Selma et al., 2008; Sardiñas et al., 2010). As an alternative to PCR-based analysis, loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000) is an easy and rapid analytical tool for the early detection of toxigenic fungi (Niessen and Vogel, 2010). In addition, LAMP was developed for the fast detection of pathogenic or allergenic fungi in the environment (Sun et al., 2010). Recently, LAMP-PCR methods using internal labeled probes have been developed for the important *Fusarium* head blight pathogens in materials of different plants (Niessen and Vogel, 2010; Abd-Elsalam et al., 2011; Niessen et al., 2012). These methods have been used to detect plant pathogenic fungi such as *Phytophthora ramorum* (Tomlinson et al., 2007), *Phytophthora sojae* (Dai et al., 2012), toxigenic *Fusarium* (Denschlag et al., 2012; Niessen et al., 2012; Almoammar et al., 2013), *Aspergillus flavus* and *Aspergillus parasiticus* (Ahmad et al., 2010), *A. flavus*, *A. parasiticus*, *Aspergillus nomius*, and *Aspergillus caelatus* (Luo et al., 2012, 2014), and *A. carbonarius* and *A. niger* (Storari et al., 2013). Based on the data presented in the literature, the LAMP-based assay may constitute a potentially valuable molecular tool for the rapid diagnosis of ochratoxigenic *Aspergillus* species in agricultural products. The aims of this study were to identify dominant ochratoxigenic *Aspergillus* species in peanuts collected from Saudi Arabia and to evaluate specific PCR and LAMP-PCR assays for identifying and quantifying OTA-producing aspergilli collected from peanuts.

MATERIAL AND METHODS

Peanut samples and fungal isolation

Thirty-four samples of peanut kernels were obtained arbitrarily from different supermarkets in 3 locations, including Mecca, Madina, and Riyadh, Saudi Arabia during 2012. Samples were stored at 4°C until fungal enumeration. Peanut kernels were used either after surface sterilization using 2% sodium hypochlorite solution or after being washed 3 times with sterile distilled water for 3 min. Five peanut kernels were randomly divided and directly seeded on Petri dishes containing malt extract agar in triplicate. Petri dishes were incubated at 25 ± 2°C and the emerging colonies were counted after 5 days. Fungal colonies were purified and then identified morphologically and microscopically according to Pitt and Hocking (1997). All isolates were maintained as slant cultures using potato dextrose agar medium at 4°C.

Ochratoxigenic ability of the isolates

Collected isolates were tested for OTA production following the method described by Téren et al. (1996) with some modifications: the isolates were grown in stationary cultures in 25-mL quantities of YES medium (2% yeast extract, 15% sucrose) at 28°C for 10 days in the dark. After incubation, 1 mL culture media was mixed with 1 mL chloroform and centrifuged at 4000 g for 10 min. The chloroform fraction was transferred to a clean tube, evaporated to dryness, and redissolved in 0.5 mL methanol. Next, OTA was detected according to the previously described procedures.

A high-performance liquid chromatography system (Shimadzu; Kyoto, Japan) was used with the fluorescence detection set at 333 nm for excitation and 477 nm for emission. The mobile phase consisted of acetonitrile:water:acetic acid (51:47:2, v/v/v) and the flow rate was 1 mL/min. An OTA stock was used to prepare 5 standard curves of peak areas versus concentration (μg/L). The OTA standard was purchased from Sigma (Ref.O-1877; St. Louis, MO, USA). Samples with an OTA level below 1μg/kg were considered to be negative controls.

Fungal isolates and DNA extraction

Aspergillius species isolates used in this study are listed in Table 1. Total genomic DNA was purified from fresh mycelia of *Aspergillus* isolates were extracted using a modified method (Bahkali et al., 2012).

Specific PCR assay

PCR amplification was carried out using 2 sets of primers: OCRA1/OCRA2 (5'-CTTCCTTAGGGGTGGCACAGC-3' and 5'-GTTGCTTTTCAGCGTCGGCC-3', respectively) for *A. ochraceus* (Patiño et al., 2005). For the PCR, 1 μL DNA sample solution was mixed with 24 μL PCR stock solution containing 2.5 μL 10X PCR buffer (Qiagen; Hilden, Germany), 2 μL 25 mM $MgCl_2$, 0.5 μL 10 mM dNTP mixture, 1 μL of each primer (20 pmol), 0.2 μL 5 U/μL *Taq* polymerase (Qiagen), and 16.3 μL sterile deionized H_2O. The amplification thermal parameters were: 4 min at 95°C, followed by 40 amplification cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 20 s. PCR products were analyzed by electrophoresis on a 1.5%

agarose gel in TAE buffer. Ethidium bromide-stained gels (0.5 µg/mL gel) were visualized under ultraviolet light and photographed using a gel-doc system (Bio-Rad Laboratories; Hercules, CA, USA).

Table 1. Ochratoxin A (OTA) production capabilities of *Aspergillus* isolates used in this study and PCR amplification products with the primer pair OCRA1/OCRA2. The 2 LAMP primer sets (AnPKS and AcPKS) were used to detect *A. niger* and *A. carbonarius*, respectively.

Aspergillius species	OTA quantity (µg/g)	PCR assay	LAMP-PCR	
		OCRA1/OCRA2	AnPKS	AcPKS
A. niger	0.00	□	■	□
A. niger	1.27	□	■	□
A. niger	0.97	□	■	□
A. niger	0.00	□	■	□
A. niger	1.90	□	■	□
A. niger	0.00	□	■	□
A. niger	0.00	□	■	□
A. ochraceus	0.88	■	□	□
A. ochraceus	0.36	■	□	□
A. ochraceus	0.79	■	□	□
A. ochraceus	1.18	■	□	□
A. ochraceus	0.93	■	□	□
A. ochraceus	1.14	■	□	□
A. carbonarius	0.00	□	□	■
A. carbonarius	1.63	□	□	■
A. carbonarius	0.23	□	□	■
A. carbonarius	1.45	□	□	■
A. carbonarius	0.00	□	□	■
A. phoenicis	0.00	□	□	□
A. flavus	0.00	□	□	□

The presence or absence of species-specific amplicon is indicated by a filled or open square, respectively, for each set of primers.

LAMP reaction

We used primer sets designed by Storari et al. (2013) with the nucleic acid sequences as shown in Table 2. The LAMP-PCR assay was carried out in a total of 25 µL reaction mixture containing 2.5 mM 10X Bst-DNA polymerase buffer, 10 µM betaine, 2.5 mM dNTPs, 10 mM MgSO$_4$, 1.6 mM of each forward inner primer and backward inner primer, 0.2 mM of each F3 and B3 primers, 0.8 mM of each loop-F and loop-B, 8 U Bst-DNA polymerase (New England BioLabs; Ipswich, MA, USA), ddH$_2$O, and 1 µL template DNA. Before incubation, 25 µM calcein and 0.5 mM MnCl$_2$ were added to each 25 µL reaction mix. Sterile distilled H$_2$O was used as a negative control template for all reactions. LAMP reactions were carried out at 63°C for 60 min using a thermal cycler (icycler; Bio-Rad). Visual inspection of the LAMP amplicons in the reaction tube were performed by adding fluorescent detection reagent before the incubation of the reaction tubes, the fluorescent signals of the solutions were detected under ultraviolet light. Direct visualization of the amplified product was achieved by electrophoresis of the LAMP reactions on 1.5% agarose gels following incubation. Gels were run at 60 V and 55 mA for 45 min and stained with 0.5 µg/mL ethidium bromide solution. The region amplified by the LAMP primers used here spanned 150 base pairs (bp). This was confirmed by the size of the smallest-sized band obtained after agarose gel electrophoresis of the LAMP reaction products. A DNA molecular weight marker of a 100-bp DNA ladder was used to determine the size of the LAMP products.

Table 2. Loop-mediated isothermal amplification primers for the detection of *Aspergillus carbonarius* (AcPKS) and *Aspergillus niger* (AnPKS) used in the present study (Storari et al., 2013).

Primer sets	Primers sequence (5'-3')	*Aspergillius* species
AcPKS FIP	GCTGCAATGCACCCGGTAG	*A. carbonarius*
	TTGAAGACGTGGAGGGCTTCT	
BIP	AAGTCACGTCAAAAGCCCTGG	
	TGATTCCTTGGGAGGTTGGTC	
Loop F	GCTGGCCGAAAAGATCGCTAA	
Loop B	CCCCTCTGCTATGAAGTCCG	
F3	TGGTGGTACGAATGCACAC	
B3	CGAAATGACAAACAGGCGGT	
AnPKS FIP	CCTGCGCCACCTTCCAAGTGC	*A. niger*
	GATTCGCCCCTCTATGTTG	
BIP	CTGATCTCAGCCACACTGGCT	
	GATCTTGGGGTTCAAGCTCTG	
Loop F	CCAGCACGGATTTTACCGATC	
Loop B	TAGAGTCGAAGATGATA CCCCCAGT	
F3	TGCATTAGGTGTTGCCCG	
B3	AGGTCATCGCGTTGAGGA	

All nucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany).

RESULTS

Cultural and morphological characteristics

Fungal colonies of *A. carbonarius* and *A. niger* on malt extract agar at 27°C were first white, and rapidly became black with conidial production. The inverse was pale yellow, and growth generated radial fissures on the agar. Malt extract agar allowed for faster growth and colonies appeared after 2-4 days. *A. carbonarius* and *A. niger* colonies were not distinguishable by eye (Figure 1).

Hyphae are composed of septate and hyaline. Conidial heads radiate initially, splitting into columns at maturity. The species is biseriate, which means that vesicles produce sterile cells known as metulae that support the conidiogenous phialides. Conidiophores are long (400-3000 μm), smooth, and hyaline, and become darker at the apex, terminating in a globose vesicle (30-75 μm in diameter) (Figure 2). Based on morphological characteristics, *A. niger* (35%), *A. ochraceus* (30%), and *A. carbonarius* (25%) were common, while *A. flavus* and *A. phoenicis* were isolated only occasionally and in low numbers (10%).

Ochratoxigenic-producing abilities of isolates assayed

Aspergillus isolates were verified for their capacity to produce OTA on YES medium. Detection of OTA in isolates studied was performed using a high-performance liquid chromatography method. Of 20 isolates, 12 (60%) produced 0.36-1.27 μg/g OTA. Most ochratoxigenic isolates were *A. ochraceus* (6), *A. carbonarius* (3), and *A. niger* (3). None of the *A. phoenicis* or *A. flavus* isolates were OTA producers. The percentage of ochratoxigenic isolates identified is also shown in Table 3. *A. ochraceus* was the main OTA producer, with 100% of the isolates able to produce this mycotoxin, followed by *A. niger* and *A. carbonarius*.
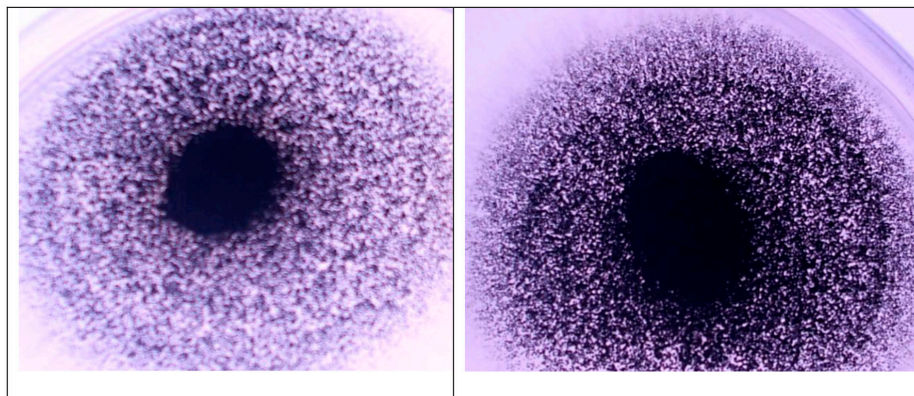
Figure 1. *Aspergillus carbonarius* (right) and *A. niger* (left) on malt extract agar 5 days after seeding.

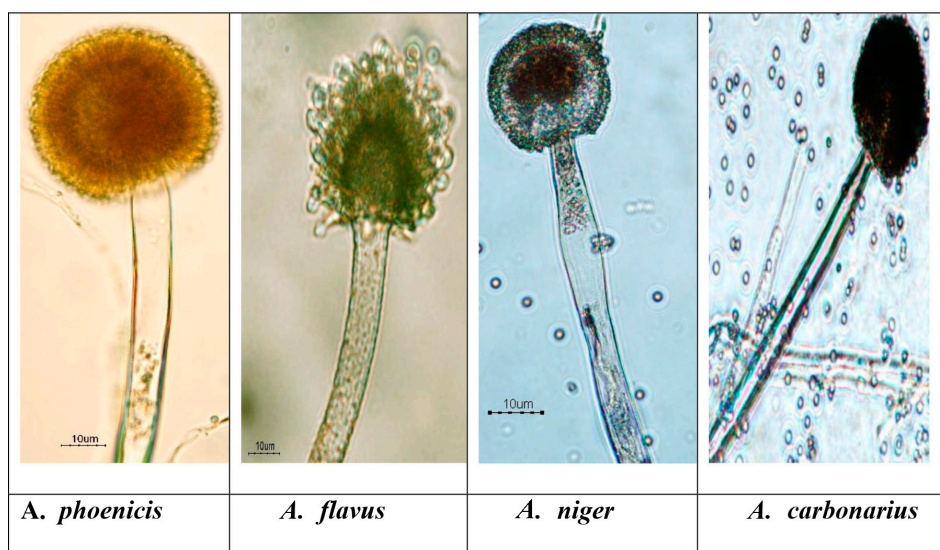| A. *phoenicis* | A. *flavus* | A. *niger* | A. *carbonarius* |

Figure 2. Microscopic characteristics of the 4 main *Aspergillius* species isolated on malt extract agar. Coenocytic, conidia, conidiospore, conidial heads, and the tiny black dots with condiospores are shown.

Table 3. *Aspergillus* strains isolated from peanuts in Saudi Arabia and the percentage of OTA-producing strains of each species.

Aspergillius species	Number of isolated strains	Ochratoxigenic strains (%)
A. niger	7	57
A. ochraceus	6	100
A. carbonarius	5	60
A. phoenicis	1	0
A. flavus	1	0
Total aspergilli isolated	20	65

Specificity of PCR and LAMP-PCR detection

A single fragment of approximately 400 bp was amplified by the OCRA1 and OCRA2 primers when genomic DNA from *A. ochraceus* isolates was used. No PCR product was observed with DNA from the *Aspergillus* isolates other than in *A. ochraceus* or in the case of other *Aspergillius* species (Figure 3). Amplification of specific gene segments was carried out at 65°C for 1 h. Amplified LAMP products were analyzed by agarose gel electrophoresis and detected by staining gels with ethidium bromide. The 2 LAMP primer sets (AcPKS and AnPKS) showed positive reactions for the DNA of all *A. carbonarius* and *A. niger* isolates used in this study (Table 1). The amplification reaction produced ladder-like pattern bands on the agarose gels, with sizes ranging from 150 bp up to the loading wells (Figure 4). The results showed that the LAMP assay was very specific for detecting and differentiating targeted species. Seven *A. niger* and 5 *A. carbonarius* isolates showed positive amplification by the LAMP assay using the AnPKS and AcPKS primers, respectively, while none of the primer sets amplified the DNA template of any closely related *Aspergillius* species, including *A. phoenicis*, *A. flavus*, and *A. ochraceus*, establishing the specificity of the LAMP test for *A. carbonarius* and *A. niger* (Figure 5).
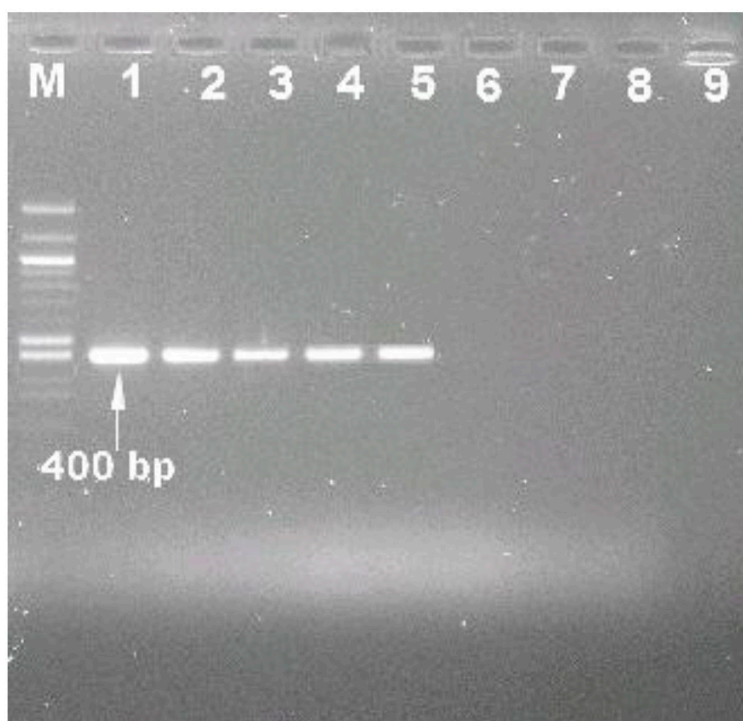

Figure 3. Electropherogram of PCR products revealed a specific band of 400 bp. *Lanes 1-5 = A. ochraceus* isolates; *lane 6 = A. flavus* isolate; *lane 7 = A. carbonarius*; *lane 8 = A. niger*; *lane 9 = A. phoenicis*. *Lane M* = 100-bp DNA molecular weight marker.
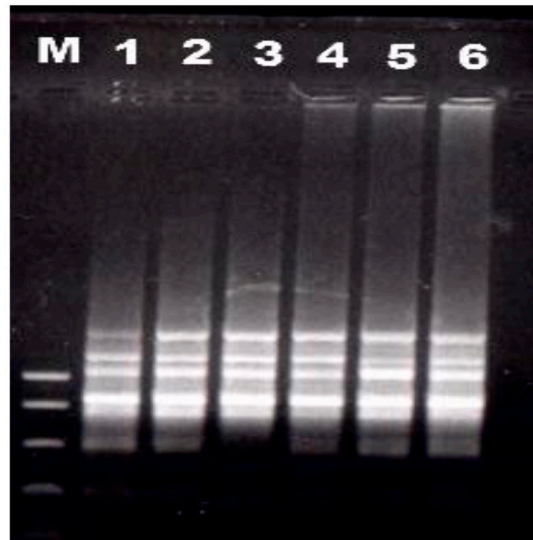
Figure 4. Comparison of template preparations for loop-mediated isothermal amplification (LAMP) reaction. *Lanes 1-6 = A. niger* isolates. LAMP products were analyzed by 1.5% agarose gel electrophoresis. The amplification showed a nucleic acid ladder-like profile. *Lane M* = 100-bp DNA molecular weight marker.
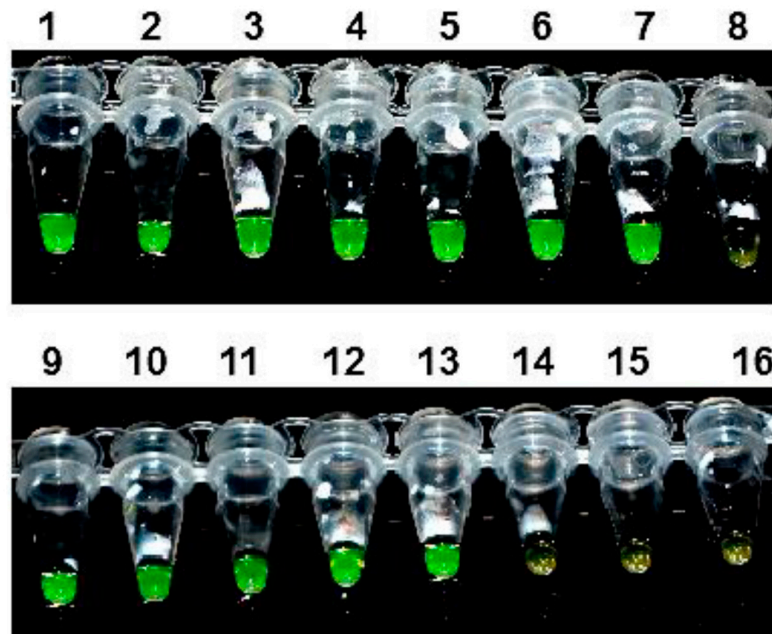

Figure 5. Specificity of LAMP-PCR assay, visual appearance of LAMP-PCR products using primer sets AnPKS and AcPKS, and naked-eye inspection under normal light. *Lanes 1-7 = A. niger* isolates; *lane 8 = A. flavus* isolate; *lanes 9-13 = A. carbonarius*; *lane 14 = A. ochraceus*; *lane 15 = A. phoenicis*; *lane 16* = non-template DNA.

DISCUSSION

The black aspergilla are the most common species associated with grapes, onions, maize, and peanuts, and also cause diseases on many grain crops such as peanut and maize seedling blight and maize kernel rot (Magnoli et al., 2006; Palencia et al., 2010). In the current study, statistical analyses revealed that the predominance of *A. niger* (35%), *A. ochraceus* (30%), and *A. carbonarius* (25%) in disinfected peanut seeds. According to the previous mycobiota surveys, peanuts are frequently contaminated by section Flavi and this infection can occur during growth, harvesting, transportation, or storage (Barros et al., 2003; Passone et al., 2010).

The OTA content of the collected isolates was determined by high-performance liquid chromatography. In the present study, *A. niger* and *A. carbonarius* are two major producers of OTA. In harmony with Accensi et al. (2001), 25 to 100% of the isolates of *A. carbonarius* are ochratoxigenic, and 0.6 to 50% of the isolates of *A. niger* are ochratoxigenic. YES medium was chosen as it was previously evaluated as an optimal substrate for the evaluation of OTA production of black aspergilli (Spadaro et al., 2010). Other studies examined the production of OTA by *Aspergillus* species on other substrates, such as Czapek yeast extract agar. In a comparison of different *Aspergillus* isolates grown on YES or Czapek yeast extract, both culture media were found useful to evaluate OTA production (Bragulat et al., 2001).

A single fragment of approximately 400 bp was amplified by the OCRA1 and OCRA2 primers when genomic DNA from *A. ochraceus* isolates was used. No PCR product was observed with DNA from the *Aspergillus* isolates other than *A. ochraceus* or for other genera. The primer pair OCRA1/OCRA2 was designed based on internal transcribed spacer sequence comparison of several strains of *Aspergillus* species (Patiño et al., 2005).

In the present study, LAMP reactions were conducted using the primer set previously designed by Storari et al. (2013) to identify and quantify 2 OTA-producing aspergilli collected from peanuts. Recently, LAMP-PCR assays were used in combination with a rapid protocol for isolating amplifiable DNA from the conidiospores of aflatoxin-producing *A. nomius* and *A. flavus*, respectively, in pure cultures and in naturally contaminated Brazil nuts (Luo et al., 2014). The LAMP assay is relatively equipment-free, only requiring a water bath or thermo block to maintain a constant temperature. It is therefore much less technically demanding compared to PCR. The present LAMP assay depends on the Bst-DNA polymerase, which is more stable than other DNA polymerases (Poon et al., 2006) such as *Taq* DNA polymerase, which is easily inhibited by biological substances. Detectable copies of DNA were obtained after only 1 h of incubation under isothermal incubation at 63°C, and visual evaluation of the results could be carried out using a simple ultraviolet lamp.

CONCLUSIONS

Our results support the hypothesis that *A. carbonarius* and occasionally *A. niger* are the main producers of OTA in peanuts. The use of specific primers for the 2 LAMP primer sets (AcPKS and AnPKS) was efficient, fast, and reproducible in the detection *A. carbonarius* and *A. niger*. The 2 LAMP assays permitted the rapid and specific identification of DNA from OTA-producing black aspergilli, as well as isolates grown in pure culture. The techniques proposed in the current study are much faster and more reliable for detecting the presence of ochratoxigenic aspergilli found in peanuts. This method can be applied for agricultural quarantine pathogens.

ACKNOWLEDGMENTS

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