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Development of loop-mediated isothermal amplification (LAMP) assay for the rapid detection of *Penicillium nordicum* in dry-cured meat products

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ABSTRACT

The need of powerful diagnostic tools for rapid, simple, and cost-effective detection of food-borne fungi has become very important in the area of food safety. Currently, several isothermal nucleic acid amplification methods have been developed as an alternative to PCR-based analyses. Loop-mediated isothermal amplification (LAMP) is one of these innovative methods; it requires neither gel electrophoresis to separate and visualize the products nor expensive laboratory equipment and it has been applied already for detection of pathogenic organisms. In the current study, we developed a LAMP assay for the specific detection of *Penicillium nordicum*, the major causative agent of ochratoxin A contamination in protein-rich food, especially dry-cured meat products. The assay was based on targeting *otapksPN* gene, a key gene in the biosynthesis of ochratoxin A (OTA) in *P. nordicum*. Amplification of DNA during the reaction was detected directly in-tube by color transition of hydroxynaphthol blue from violet to sky blue, visible to the naked eye, avoiding further post amplification analyses. Only DNAs isolated from several *P. nordicum* strains led to positive results and no amplification was observed from non-target OTA and non OTA-producing strains. The assay was able to detect down to 100 fg of purified targeted genomic DNA or 10^2 conidia/reaction within 60 min. The LAMP assay for detection and identification of *P. nordicum* was combined with a rapid DNA extraction method set up on serially diluted conidia, providing an alternative rapid, specific and sensitive DNA-based method suitable for application directly "on-site", notably in key steps of dry-cured meat production.

1. Introduction

Penicillium nordicum is a widely distributed contaminant of protein-rich food. It is most often found on dry-cured meat products (cured ham, salami), salted fish and, occasionally, on cheese and jam. It is an important and consistent producer of ochratoxin A (OTA), which can be detected in the *P. nordicum* mycelium and spores and also in food products contaminated by *P. nordicum* (Frisvad and Samson, 2004). OTA is a potent mycotoxin, and it has nephrotoxic, nephrocarcinogenic, teratogenic, neurotoxic and immunotoxic activities (EFSA, 2006; Marin-Kuan et al., 2008; Van Egmond, 2002). Moreover, it is involved in porcine and chicken nephropathy (Stoev et al., 2010) and is suspected to be an important etiological factor in human Balkan endemic nephropathy, as well as in the occurrence of tumors of urogenital tract (Pfohl-Leszkowicz et al., 2002).

Current diagnostic systems for toxigenic fungi are based mainly on microbial isolation and subsequent identification through morphological and PCR-based methods. These approaches require fully equipped laboratory and usually are expensive and time consuming. To overcome these limitations the development of powerful diagnostic tools for rapid, simple, and cost-effective detection of food-borne fungi of food safety concern is advantageous, especially if applicable directly "on-site" in food chain production. Recently, Niessen et al. (2013) reviewed the literature available on several developed isothermal nucleic acid amplification methods for food-borne bacterial pathogens and fungal contaminants as an alternative to PCR-based analyses. Loop-mediated isothermal amplification (LAMP) is a molecular method widely exploited for the detection of pathogenic organisms as it has the advantage of requiring neither gel electrophoresis to visualize amplified products nor expensive laboratory equipment. LAMP is a simple and rapid nucleic acid amplification method which employs a strand-displacement DNA polymerase and produces amplicons containing a mixture of stem-loop DNAs with various stem lengths and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand, without the need of thermal denaturation (Notomi et al., 2000). Amplification products generated by LAMP can be visualized in different ways besides traditional gel electrophoresis: increase of turbidity due to precipitation of magnesium

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pyrophosphate (Mori et al., 2001); increase of fluorescence by addition of DNA intercalating dyes (Hill et al., 2008) or complexometric dyes; color change when the complexometric dyes attach divalent metal ions (Goto et al., 2009; Tomita et al., 2008). Hydroxynaphthol blue (HNB), a metal ion indicator leading to a color change of reaction solution from violet to sky blue, was recently suggested by Goto et al. (2009) to detect DNA amplification in LAMP. The LAMP reaction results in large amounts of pyrophosphate ion byproduct; these ions react with Mg^{2+} ions to form the insoluble product magnesium pyrophosphate. Since Mg^{2+} ion concentration decreases as the LAMP reaction progresses, the LAMP reaction can be quantified by measuring the Mg^{2+} ion concentration in the reaction solution. Therefore, HNB is used as an indicator for the LAMP reaction and it can be added in pre-reaction solution, with the important advantages of increasing limits of detection for Mg^{2+} and avoiding the opening of reaction tubes with reduction of cross-contamination risk (Goto et al., 2009). The LAMP method has been widely applied for the detection of several human (Parida et al., 2008), animal (Cardoso et al., 2010) and plant pathogens (Kuan et al., 2010). Recently, it was utilized also for detecting fungal contamination in pepper and paprika powder (Zhang et al., 2014) or to detect specific mycotoxin-producing fungi, such as *Fusarium graminearum*, *Aspergillus carbonarius*, *A. niger* and three species of *Aspergillus* section *Flavi* (Luo et al., 2012; Niessen and Vogel, 2010; Storari et al., 2013). The main objective of the present study was to develop a LAMP assay for easy, rapid, highly sensitive, and specific detection of *P. nordicum*, in order to allow the identification of potential OTA risk contamination directly "on-site" in food production chains. Based on our knowledge, this is the first LAMP assay developed for the detection of *P. nordicum*.

2. Material and methods

2.1. Fungal cultures and DNA extraction

A collection of 41 strains from the Agri-Food Toxigenic Fungi Culture Collection (www.ispa.cnr.it/Collection/) of the Institute of Sciences of Food Production (ISPA-CNR, Bari, IT) were used in this work (Table 1). The strains were selected among different genera and species, among which *Aspergillus niger* ITEM 9568, *A. welwitschiae* ITEM 7097, *A. carbonarius* ITEM 5010, *A. lacticoffeatus* ITEM 7559, *A. sclerotioniger* ITEM 7526 , *Penicillium verrucosum* (3 strains) and *P. nordicum* (13 strains) were OTA producers. Working cultures were maintained on PDA medium (potato extract 0.4% w/v, dextrose 2% w/v, agar 1.5% w/v) at 25 °C for 5–7 days and stored as conidial suspension in 15% v/v glycerol at −20 °C. Total genomic DNA was extracted from mycelium grown in Wickerham medium (dextrose 4% w/v, peptone 0.5% w/v, yeast extract 0.3% w/v, malt extract 0.3% w/v, sterilized at 110 °C for 15 min) at 25 °C for 72–96 h. Mycelium was collected by filtration on Whatman paper (No. 4) and DNA was extracted from 100 mg of dried mycelium by using the Wizard® Magnetic DNA Purification System for Food kit (Promega, Madison, USA), according to manufacturer's instructions. Quality and concentration of DNA were determined by agarose gel electrophoresis and spectrophotometric analysis using NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, USA).

2.2. Primer design and LAMP reaction

The target gene selected for LAMP assay was the *otapksPN* gene (GenBank accession no. AY557343.2), coding for a polyketide synthase (PKS) previously reported by Geisen et al. (2006) to be involved in OTA biosynthesis in *P. nordicum*. In particular, primers were designed on the inter-domain region between the beta-ketoacyl synthase (KS) and the acyl transferase (AT) domains (position from nucleotide 9209 to 9613) by using the open access PrimerExplorer V.4 software tool (http://primerexplorer.jp/e/) provided by Eiken Chemical Co., Ltd. (Tokyo, Japan). Primers designed were outer primers F3-ID21 and B3-ID21 and inner primers FIP-ID21 and BIP-ID21 (Table 2). Fig. 1 shows the schematic position of LAMP primers within the target region. The selected primers were tested for similarities with other nucleotide sequences available in GenBank databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLASTN algorithm.

LAMP reaction conditions, temperature and concentration of reagents were optimized. In order to select the optimal temperature the following reaction conditions were used: 2.5 μl 10× thermopol buffer (New England Biolabs, Hertfordshire, UK), 8 mM $MgSO_4$ (Sigma-Aldrich, Munich, Germany), 0.8 M betaine (Sigma-Aldrich, Munich, Germany), 1.6 mM dNTPs (Euroclone, Milano, Italy), 100 μM of HNB (Sigma-Aldrich, Munich, Germany), 1.6 μM each of FIP and BIP primers (HPLC-purified), 0.2 μM each of F3 and B3 primers, 1 μl of purified *P. nordicum* ITEM 9634 DNA (20 ng), 1 μl (8 units) of Bst DNA polymerase (New England Biolabs) and sterile deionized water up to 25 μl. In order to determine the optimal temperature the reaction mix was incubated using an Eppendorf Mastercycler ep Gradient S (Eppendorf AG, Germany) in a temperature gradient between 65 °C and 70 °C for 60 min, followed by a final heating at 85 °C for 2 min to

Table 1
Fungal isolates used during the current study and respective OTA production (+/−) and amplification results (+/−) in LAMP assays.

Genus	Species	Strain	OTA	LAMP
Alternaria	*alternata*	ITEM 15921	−	−
Aspergillus	*niger*	ITEM 9568	+	−
	niger	ITEM 5218	−	−
	niger	ITEM 4501	−	−
	welwitschiae	ITEM 10353	−	−
	welwitschiae	ITEM 7097	+	−
	aculeatus	ITEM 7046	−	−
	tubingensis	ITEM 7040	−	−
	carbonarius	ITEM 5010	+	−
	flavus	ITEM 7826	−	−
	flavus	ITEM 7528	−	−
	lacticoffeatus	ITEM 7559	+	−
	sclerotioniger	ITEM 7526	+	−
Fusarium	*sporotrichioides*	ITEM 7637	−	−
Penicillium	*nalgiovense*	ITEM 16169	−	−
	salamii	ITEM 15291	−	−
	caseifulvum	ITEM 7543	−	−
	expansum	ITEM 7545	−	−
	rugulosum	ITEM 9587	−	−
	gladioli	ITEM 16185	−	−
	brevicompactum	ITEM 7563	−	−
	variabile	ITEM 9588	−	−
	griseofulvum	ITEM 7569	−	−
	olsoni	ITEM 9586	−	−
	chrysogenum	ITEM 9574	−	−
	verrucosum	ITEM 4518	+	−
	verrucosum	ITEM 4519	+	−
	verrucosum	ITEM 7539	+	−
	nordicum	ITEM 9634	+	+
	nordicum	ITEM 13080	+	+
	nordicum	DTO 098 − F7	+	+
	nordicum	DTO 279 − F8	+	+
	nordicum	DTO 279 − F7	+	+
	nordicum	DTO 279 − F4	+	+
	nordicum	DTO 105 − H2	+	+
	nordicum	DTO 279 − G1	+	+
	nordicum	BFE 517	+	+
	nordicum	BFE 520	+	+
	nordicum	BFE 541	+	+
	nordicum	BFE 542	+	+
	nordicum	BFE 856	+	+

Table 2
List of LAMP primers used in this study.

Primer name	5′–3′ oligonucleotide sequence
F3-ID21	TTCCGGCATCATTCATCCAA
B3-ID21	CCGGAGTCTCCAGCTATGG
FIP-ID21	AGTCAGACGGCCCACAAATCCTCCTGGGCACTGATACGGT
BIP-ID21	TCTTCAAATGGTGGCGGCGTCTACGGAGGAACAGTTTCGC