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Development of a PCR protocol to detect patulin producing moulds in food products

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ABSTRACT

Patulin is a secondary toxic metabolite with important health effects. Several mould species of *Penicillium* and *Aspergillus* genera associated with patulin production have been detected in food products. Thus, specific and sensitive methods to detect patulin producing moulds are needed. The aim of this work was to develop a polymerase chain reaction (PCR) method to detect patulin producing moulds in food. 34 patulin producing and 30 non-producing strains belonging to the main species usually reported in food products were used. Patulin production was firstly evaluated by mycellar electrokinetic capillary electrophoresis and high-pressure liquid chromatography-mass spectrometry in all tested strains. Biosynthesis was also used to develop PCR primers derived from the genes involved in patulin. By means of a primer pair based on the isoepoxydon dehydrogenase (*idh*) gene, a 496-bp amplicon was specifically detected in all the mould strains previously confirmed as patulin producing, regardless of their genus and species. With the developed method it was possible to detect down to 0.5 ng of pure DNA from producing strains and from 1.8×10^2 to 2.7×10^3 conidia g^{-1} in artificially inoculated foods. No relevant PCR inhibition due to food matrices was observed. The PCR protocol developed could be considered as an appropriate tool to detect patulin producing moulds in food products.

1. Introduction

Patulin is a tetraketide mycotoxin featuring antibiotic, toxic, mutagenic, and carcinogenic properties (Beretta, Gaiaschi, Galli, & Restani, 2000; López-Díaz & Flannigan, 1997; Smith & Moss, 1985). This mycotoxin is a secondary metabolite produced by different *Penicillium* species, such as *Penicillium expansum* and *Penicillium griseofulvum* (Dombrink-Kurtzman & Blackburn, 2005; Frisvad, Smedsgaard, Larsen, & Samson, 2004; Niessen, 2007), and *Aspergillus* species, including *Aspergillus clavatus*, *Aspergillus giganteus*, and *Aspergillus terreus* (Steiman, Seiglle-Murandi, Saye, & Krivobks, 1989; Varga et al., 2003). Because of the growth of these mould species, patulin can be found in apples and its derived products, and occasionally in other fruits such as pears, apricots, peaches, and grapes, where it is involved in the rotting process (Cheraghali et al., 2005). Furthermore, *Aspergillus* and *Penicillium* species isolated from dry-cured meat products and ripened cheeses have been reported as patulin producing mould (Erdogan, Gurses, & Sert, 2003; Erdogan & Sert, 2004; Martín, Jurado, Rodríguez, Núñez, & Córdoba, 2004).

To prevent patulin occurrence in food, producing moulds should be detected early in the food chain (Barreira, Alvito, & Almeida, 2010; Dvali, Maksimenko, Eller, & Tutel'ian, 1985; Majerus, Hain, & Kölb, 2008; Marín et al., 2011; Morales et al., 2008; Tangni et al., 2003; Yuan, Zhuang, Zhang, & Liu, 2010). The demand is thus to design rapid and sensitive methods that detect these moulds early enough. The methods applied in the identification of such moulds require culture isolation and morphological and physiological characterization (Gourama & Bullerman, 1995). They are time-consuming, labor-intensive, and often need mycological expertise (Shapira, Paster, Menasherov, Mett, & Salomon, 1996). Besides, plate count techniques do not seem to detect dead fungi, which would indicate past contamination by a food product (Marek, Annamalai, & Venkitanarayanan, 2003).

Polymerase chain reaction (PCR) has been applied as an alternative technique that replaces microbiological and chemical methods in the detection and identification of some toxigenic moulds in food (Paterson, 2006). PCR methods have been developed to detect aflatoxigenic and ochratoxigenic moulds isolated from food products (Bogs, Battilani, & Geisen, 2006; Del Fiore et al., 2010; Patiño, González-Salgado, González-Jaén, & Vázquez, 2005; Richard, Houtte, Bouchart, & Garon, 2009). In the detection of patulin producing moulds, several conventional PCR protocols have been designed by using the isoepoxidon

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dehydrogenase (*idh*) gene. This enzyme is involved in the biosynthetic pathway of patulin in the *Penicillium* and *Aspergillus* genera (Dombrink-Kurtzman, 2006, 2008; Paterson et al., 2000; Puel, Galtier, & Osawald, 2010). Some of these PCR protocols were designed to only detect specific species of *Penicillium* genus (Dombrink-Kurtzman, 2006, 2008; Paterson et al., 2000; Puel et al., 2010) or *Aspergillus* genus (Varga et al., 2003). However no protocols have been reported to detect patulin producing moulds regardless of genus and species.

In the development of a PCR protocol for the detection of toxigenic moulds in food products, an evaluation of the influence of food-based substances on sensitivity must be carried out. Certain food components, such as fat, polysaccharides, polyphenols, and other secondary compounds, have been reported as major obstacles for an efficient amplification in PCR (Dickison, Kroll, & Grant, 1995).

This paper aims to provide a sensitive and specific PCR method to detect patulin producing mould strains in food products.

2. Materials and methods

2.1. Mould strains and culture conditions

The mould strains used in this study are listed in Table 1. The moulds were grown on Malt Extract Agar (2% malt extract, 2% glucose, 0.1% peptone, and 2% agar) for 20 days at 25 °C. Conidia were then collected in 5 mL of sterile deionized water containing 10% (v v^{-1}) of glycerol (Scharlau Chemie S.A., Barcelona, Spain) and stored at −80 °C until being used.

The conidia, inoculated in food samples, were first extracted from three 20-day-old plates of Malt Extract Agar, as described above. The conidial suspension was diluted in sterile deionized water and quantified by microscopy, using a Neubauer counting chamber.

For DNA and mycotoxin extractions, mould strains were grown on Malt Extract Agar at 25 °C for 4 and 15 days, respectively.

To evaluate the sensitivity of the PCR method in food matrices, 200 μL of previously prepared conidia suspensions were artificially inoculated into three non-sterile food groups (cooked products, ripened food, and fruits) in order to reach levels ranging from about 10 to 10^6 conidia g^{-1}. Cooked ham, mortadella, and cooked turkey breast were inoculated with *Penicillium commune* CBS 311.48, while dry-fermented sausage "salchichón" and dry-cured ham were inoculated with *P. griseofulvum* CBS 110.420 and ripened cheese with *Penicillium camemberti* CECT 2267 and *P. commune* CBS 341.59. Finally, in the fruit group, the apples and pears were inoculated with *P. expansum* CECT 2280, and the peach with *P. griseofulvum* CBS 110.420.

Non-inoculated control samples were also prepared for each food. All the assays were carried out in triplicate.

2.2. Examination of patulin production

Twelve of the 64 reference strains were marked by their respective Culture Collections as patulin producing moulds (Table 1). In this work, as mentioned above, patulin production was tested in all the mould strains. Because of this, patulin production was analyzed by micellar electrokinetic capillary electrophoresis (MECE), based on the absorbance spectrum ranging between 190 and 600 nm (Martín et al., 2004), whereas the analysis also included high-pressure liquid chromatography-mass spectrometry (HPLC-MS), with the achievement of full MS spectra after atmospheric pressure chemical ionization (Sosa et al., 2002).

2.3. DNA extraction from pure cultures and from food matrices

Grown mycelium was scraped off the agar by using a spatula. About 50 mg of mycelium isolated from each strain were used for total DNA extraction, using the method previously described by Sánchez, Rodríguez, Casado, Martín, and Córdoba (2008).

To quantify the extracted DNA, 5 μL of each DNA sample were analyzed in 1% (w v^{-1}) agarose gel electrophoresis by submerged electrophoresis, using 1XTAE buffer at 90 V for 15 min. Gels were stained with ethidium bromide (0.5 μg mL^{-1}, Sigma Aldrich Co., St. Louis, Missouri, USA), and DNA visualized by UV transillumination G-Box of Syngene (Synoptics group, Frederick, Maryland, USA). Besides, they were photographed by means of the camera and software equipment, GeneSnap and GeneTools, by Syngene. A standard equation relating the absorbance values and the concentrations of DNA standards (0.25 μg μL^{-1}, Roche Diagnostics, Indianapolis, Indiana, USA) was then generated in order to estimate the concentration (ng) of purified DNA. In addition, the quality of extracted DNA was determined spectrophotometrically in a Biophotometer Eppendorf (Eppendorf AG, Hamburg, Germany), based on the ratio at 260:280.

For the DNA extraction from food samples, 5 g of the inoculated foods were mixed with 10 mL of Tris–HCl buffer (pH 8.0), and then homogenized in a filter bag BagPage (Interscience, Paris, France) by means of a stomacher machine (IUL Instruments, Barcelona, Spain). The filtrate obtained within each bag was transferred to a sterile tube, and centrifuged first at 2500 rpm for 30 s, and then, at 13,000 rpm for 10 min. The pellets were resuspended in 100 μL of previously boiled (95 °C for 10 min) sterile deionized water to release the DNA, and cooled on ice for 10 min. Next, 200 μL of cetyltrimethylammonium bromide (CTAB) extraction buffer (20 g L^{-1} CTAB, 1.4 M NaCl, 20 mM Na_2EDTA, 0.1 M Tris–HCl, pH 8.0), 400 μL of PBS-buffer (0.15 M NaCl, 0.01 M sodium phosphate buffer, 0.05% Tween 20, pH 7.4), 20 μL of proteinase K (10 mg mL^{-1}, Sigma Aldrich Co), and 200 μL of liticase (4 U mL^{-1}, Sigma Aldrich Co.) were added before incubating at 65 °C for 1 h. Samples were then washed, shaken once in 600 μL of chloroform, and centrifuged at 13,000 rpm for 20 min, and the supernatant was transferred to a new tube. After that, 20 μL of an RNase solution (10 mg mL^{-1}, Sigma Aldrich Co.) were added before incubating at 37 °C for 1 h. Then, the same volume of chloroform was added, vortexed, and centrifuged at 13,000 rpm for 5 min. Finally, the aqueous phase was processed according to the EZNA Fungal DNA Mini Kit (Omega Bio-Teck, Doraville, Georgia, USA), starting from DNA precipitation by adding 600 μL of cold isopropanol. In the final step, DNA was eluted in 25 μL of previously warmed elution buffer (at 65 °C) that was then kept at −20 °C until used as template for PCR.

For the PCR amplification, three replicates of 5 μL of DNA, extracted from each inoculated food, were assayed per run. The non-inoculated negative control was also performed in triplicate. Results were then visualized on 2% agarose gels, as described previously.

2.4. PCR detection of patulin producing moulds

DNA from three patulin producing strains, chosen at random (*Penicillium dipodomyicola* IBT 26223, *P. griseofulvum* IBT 14319, and *P. expansum* CECT 2278), was amplified by using three primer pairs: PEF/PER, based upon the polygalacturonase gene (Yao et al., 1999; GenBank Accession number AF047713), IDH1/IDH2 from the *idh* gene (Paterson, 2007; GenBank Accession number AF006680), and IAO3844/IAO4579 based on the isoamyl alcohol oxidase *(iao)* gene (Dombrink-Kurtzman, 2008; GenBank Accession number EF202832). PCR conditions reported by former authors were applied, and all the assays were then performed in triplicate.

The primers that specifically amplified all the patulin producing strains in all the assays were selected for further analysis. Thus, the

Table 1
Patulin production and PCR data with the specific primers FC2 and IDH2 of the reference mould strains used in this study.

Species designation	Strain reference	Information by type culture collections[e]	Patulin production as detected by MECE and/or HPLC-MS[f]	PCR result (FC2/IDH2)[g]
Aspergillus terreus	CBS 601.65[a]	+	+	+
Penicillium carneum	CBS 468.95	+	+	+
Penicillium dipodomyicola	IBT 26223[b]	+	+	+
Penicillium dipodomyicola	CBS 110.425	+	+	+
Penicillium expansum	CECT 2278[c]	+	+	+
Penicillium expansum	CECT 20140	+	+	+
Penicillium expansum	CECT 2279	+	+	+
Penicillium expansum	CECT 2280	+	+	+
Penicillium griseofulvum	IBT 14319	+	+	+
Penicillium griseofulvum	CBS 485.84	+	+	+
Penicillium griseofulvum	CBS 110.420	+	+	+
Penicillium griseofulvum	CECT 2919	+	+	+
Aspergillus flavus	CECT 2684	U	+	+
Aspergillus oryzae	CECT 20168	U	+	+
Aspergillus oryzae	CECT 2095	U	+	+
Aspergillus tamarii	CBS 575.65	U	+	+
Aspergillus tamarii	CBS 109.63	U	+	+
Emericella quadrilineata	CBS 235.65	U	+	+
Emericella rugulosa	CBS 133.60	U	+	+
Emericella variecolor	CBS 135.55	U	+	+
Penicillium aurantiogriseum	CECT 2918	U	+	+
Penicillium aurantiogriseum	CBS 112021	U	+	+
Penicillium camemberti	CECT 2267	U	+	+
Penicillium camemberti	FHSC-1[d]	U	+	+
Penicillium camemberti	CBS 273.97	U	+	+
Penicillium commune	CBS 311.48	U	+	+
Penicillium commune	CBS 341.59	U	+	+
Penicillium dipodomyicola	CBS 110426	U	+	+
Penicillium melanoconidium	CBS 64195	U	+	+
Penicillium melanoconidium	CBS 109605	U	+	+
Penicillium polonicum	FHSC-2	U	+	+
Penicillium polonicum	CBS 639.95	U	+	+
Penicillium polonicum	CBS 112490	U	+	+
Penicillium verrucosum	FHSC-3	U	+	+
Aspergillus awamorii	CBS 101702	U	−	−
Aspergillus flavus	CECT 2687	U	−	−
Aspergillus flavus	IBT 3696	U	−	−
Aspergillus flavus	CBS 573.65	U	−	−
Aspergillus flavus	CBS 120.62	U	−	−
Aspergillus foetidus	CBS 101708	U	−	−
Aspergillus fumigatus	CBS 192.65	U	−	−
Aspergillus niger	CECT 20157	U	−	−
Aspergillus ochraceoroseus	CBS 101887	U	−	−
Aspergillus ochraceus	CBS 589.68	U	−	−
Aspergillus oryzae	CECT 2094	U	−	−
Aspergillus parasiticus	CECT 2688	U	−	−
Aspergillus parasiticus	CECT 2682	U	−	−
Aspergillus parasiticus	CECT 2681	U	−	−
Aspergillus parasiticus	CBS 571.65	U	−	−
Aspergillus toxicarius	CBS 822.72	U	−	−
Aspergillus tubingensis	CECT 20543	U	−	−
Aspergillus tubingensis	CECT 20545	U	−	−
Aspergillus versicolor	CECT 2664	U	−	−
Aspergillus versicolor	CECT 2903	U	−	−
Aspergillus versicolor	CECT 2814	U	−	−
Emericella heterothallica	CBS 488.65	U	−	−
Emericella nidulans	CBS 465.65	U	−	−
Penicillium aurantiogriseum	CECT 2264	U	−	−
Penicillium commune	CBS 247.32	U	−	−
Penicillium nordicum	CBS 110769	U	−	−
Penicillium verrucosum	CECT 2906	U	−	−
Penicillium verrucosum	CBS 323.92	U	−	−
Penicillium viridicatum	CECT 2320	U	−	−
Rhizopus oryzae	CBS 607.68	U	−	−

[a] CBS: Centraalbureau voor Schimmelcultures in The Netherlands.
[b] IBT: Type Culture Collection of the Department of Biotechnology from the Technical University of Denmark.
[c] CECT: Spanish Type Culture Collection.
[d] FHSC: Food Hygiene and Safety Collection.
[e] (+): producing-patulin based on Culture Collections information; (U): information about patulin production from Culture Collections is unknown.
[f] patulin production analyzed by MECE and HPLC-MS is scored as (+), positive, according to detection by both methods or one of them, or (−) not detected.
[g] (+): an amplicon of 496 bp is observed in agarose gel; (−): an amplicon of 496 bp is not observed in agarose gel.

Table 2
Sequences of PCR primers used for the detection of the isoepoxidon dehydrogenase (*idh*) gene in patulin producing moulds.

Primer pairs	Sequence nucleotides (5′-3′)	Positions[a]	Expected PCR product (bp)
IDH1	CAATGTGTCGTACTGTGCCC	2195	504
RCA1	CATTGAGCGGATGCCCTT	2682	
IDH1	CAATGTGTCGTACTGTGCCC	2195	480
RCB1	CGGATGCCCTTCGGGCC	2675	
FC2	CGATGTTGCTAGCAAAGACG	2297	496
IDH2R	ACCTTCAGTCGCTGTTCCTC	2774	

[a] Positions are in accordance with the published sequences of the *idh* gene of *Penicillium urticae* (GeneBank Accession number AF006680).

PCR products obtained with the selected primer pair (IDH1/IDH2) were purified by using the MinElute® PCR Purification Kit, and according to the manufacturer's instructions (QIAGEN, Hilden, Germany). They were submitted for sequencing at Instituto de Biomedicina (CSIC, Valencia, Spain). The PCR product sequences were compared with *idh* gene sequences stored on the Blast database, by NCBI (www.ncbi.blast.com), and then analyzed to design three new primers (Table 2) by means of the Primer Express software (Applied Biosystems, Foster City, California, USA).

The primers were tested with six patulin producing mould strains (*P. camemberti* CECT 2267, *P. dipodomyicola* IBT 26223, and CBS 110.425, *P. expansum* CECT 2278, *P. griseofulvum* CBS 485.84, and *Penicillium polonicum* FHSC-2) in order to select the most specific primer pair. Reactions were performed in a total volume of 50 μL, containing 5 μL of 10X Mg free PCR buffer, 3 μL of $MgCl_2$ (50 mM), 1 μL of a deoxynucleotide (dNTP) mix (10 mM), 2 μL of each primer (10 mM), 0.5 μL of *Taq* polymerase (2 U μL^{-1}, Finnzymes, Espoo, Finland), and 5 μL of genomic DNA (10 ng). The reaction mixtures were incubated in a programmable thermal cycler, Mastercycler epgradient from Eppendorf AG, using the following amplification conditions: the initial denaturation step at 94 °C for 4 min; 30 cycles at a melting temperature of 94 °C for 1 min, an annealing temperature of 52 °C for 1 min, and an extension temperature of 72 °C for 1 min. A final extension of 5 min at 72 °C was included.

The amplification products were analyzed in 2% (w v^{-1}) agarose gels by using the 1XTAE buffer at 80 V for 1 h. Gels were stained with ethidium bromide, and the products visualized and photographed as described previously. A DNA molecular size marker of 2.1–0.15 kbp from Roche Diagnostics was used to determine the size of the PCR products.

The specificity of the selected PCR protocol for the detection of patulin producing moulds was evaluated in triplicate by using all the mould strains investigated.

2.5. Sensitivity of PCR protocol

To accurately measure sensitivity of the designed PCR protocol, several DNA concentrations of the patulin producing strain *P. expansum* CECT 2278, ranging from 50 ng to 0.25 ng, were assayed by using the primer pair FC2/IDH2. The detection limit of the PCR method was defined as the lowest DNA concentration of *P. expansum* CECT 2278 detected in all reactions.

The sensitivity of the PCR method was also tested in the presence of a high amount of DNA from a non-producing strain. Thus, 50 ng of DNA from *Penicillium nordicum* CBS 101.769 were mixed with different amounts of DNA (ranging from 50 ng to 0.5 ng) from the producing strain *P. griseofulvum* CBS 110.420.

Furthermore, sensitivity was tested in non-sterile food products artificially inoculated with conidia of patulin producing strains, as described in Section 2.1 above. The detection limit of the developed PCR method was estimated as the lowest number of conidia inoculated in each tested food, showing amplification in PCR.

As a last test, the influence of food components on PCR was measured by using 5 μL of pure DNA from the patulin producing *P. commune* CBS 311.48, *P. griseofulvum* CBS 110.420, and *P. expansum* CECT 2280, mixed in different volumes (1, 2, 3, 4, and 5 μL) of DNA extracted from the corresponding non-inoculated food. The products resulting from the PCR reactions were compared with those obtained by only amplifying DNA from pure strains.

3. Results

3.1. Production of patulin

All the mould strains marked by their respective Culture Collections as patulin producing (12 strains) were confirmed by

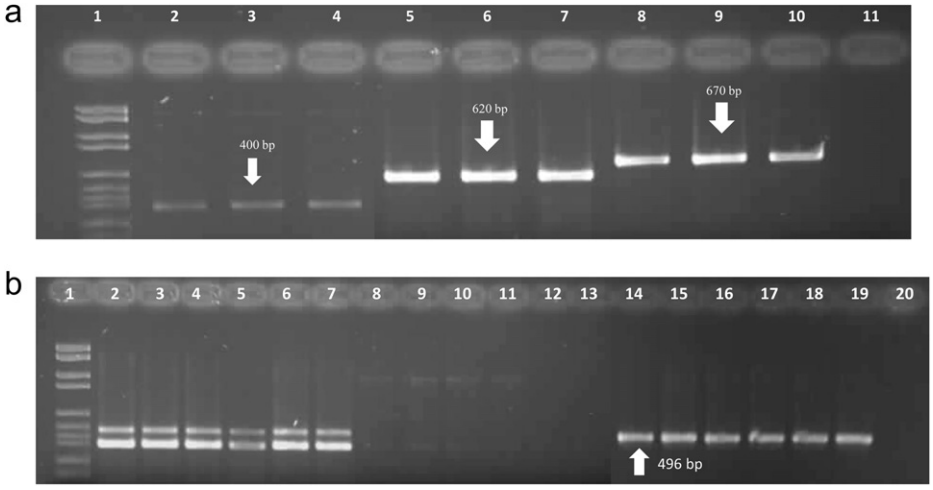

Fig. 1. (a) Agarose gel electrophoresis of PCR products obtained with primer pairs PEF/PER (lanes 2, 3, and 4), IDH1/IDH2 (lanes 5, 6, and 7), and IAO3844/IAO4579 (lanes 8, 9, and 10), and genomic DNA from *Penicillium dipodomyicola* IBT 26223 (lanes 2, 5, and 8), *Penicillium griseofulvum* IBT 14319 (lanes 3, 6, and 9), and *Penicillium expansum* CECT 2278 (lanes 4, 7, and 10). Lane 1: DNA molecular size marker of 2.1–0.15 kbp. Lane 11: negative control using DNA from non-producing *Penicillium aurantiogriseum* CECT 2320. (b) Agarose gel electrophoresis of PCR products obtained with primer pairs IDH1/RCA1 (lanes 2, 3, 4, 5, 6, and 7), IDH1/RCB1 (lanes 8, 9, 10, 11, 12, and 13), and FC2/IDH2 (lanes 14, 15, 16, 17, 18, and 19). Lane 1: DNA molecular size marker of 2.1–0.15 kbp. Lanes 2, 8, and 14: *Penicillium camemberti* CECT 2267. Lanes 3, 9, and 15: *Penicillium expansum* CECT 2278. Lanes 4, 10, and 16: *Penicillium dipodomyicola* IBT 26223. Lanes 5, 11, and 17: *P. dipodomyicola* CBS 110.425. Lanes 6, 12, and 18: *Penicillium griseofulvum* CBS 485.84. Lanes 7, 13, and 19: *Penicillium polonicum* FHSC-2. Lane 20: negative control using DNA from non-producing *Penicillium aurantiogriseum* CECT 2320.

MECE and/or HPLC-MS (Table 1). In addition, 22 strains lacking the information on patulin production from the Culture Collections, demonstrated production of this mycotoxin by MECE and/or HPLC-MS (Table 1). The remaining 30 strains did not show production of patulin in MECE or HPLC-MS analyses (Table 1).

3.2. PCR detection of patulin producing moulds

PCR products in the expected sizes (bp) were obtained by using the three primer pairs PEF/PER, IDH1/IDH2, and IAO3844/IAO04579 in the three patulin producing moulds assayed (Fig. 1a).

The sequence of PCR products generated by the primer pair IDH1/IDH2 showed 100% of similarity when compared with the *idh* gene sequences from the NCBI database (GenBank Accession number AF006680). However, the similarity of amplicon sequences obtained with the primer pairs PEF/PER and IAO3844/IAO04579 based on polygalacturonase and *iao* genes (GenBank Accession numbers AF047713 and EF202832) was only about 80%. For the development of a PCR method able to detect patulin producing strains, regardless of genus and species, only PCR products obtained with the primer IDH1/IDH2 were selected for further analysis. From the analysis of the sequences of the former PCR products and the sequences from the NCBI database (GenBank Accession number AF006680), three new primers, one forward (FC2) and 2 reverse (RCA1 and RCB1), were then designed by using the sequence from the conserved areas of the *idh* gene. These primers were used in combination with both IDH1 and IDH2 for the achievement of three primer pairs (Table 2).

With primer pair IDH1/RCA1, two very similar amplicons of approximately 504 bp were detected in all the patulin producing strains (Fig. 1b). When using the primer pair IDH1/RCB1, a very weak PCR product of about 480 bp was obtained in 4 of the 6 tested patulin producing strains (Fig. 1b). Finally, the primer pair FC2/IDH2 yielded a specific amplicon of 496 bp in all the patulin producing strains (Fig. 1b). As a result, the last primer pair was chosen to detect patulin producing strains in the 64 moulds used. A 496-bp-amplicon (Table 1) was detected in 34 moulds, including producing mould strains belonging to the *Aspergillus* and *Penicillium* genera. The remaining strains did not show it. This amplicon detection matched the patulin production confirmed by either analytical technique, MECE or HPLC-MS (Table 1).

3.3. Sensitivity of PCR protocol

The sensitivity of the performed PCR was tested by using serial dilutions of the *P. expansum* CECT 2278 DNA obtained from a pure culture. Amplification with the primers FC2 and IDH2 was detected down to 0.5 ng of DNA from the producing strain (Fig. 2a). Thus, with the described experimental set-up it was possible to detect down to 0.5 ng of DNA. In addition, the PCR 496-bp-product was detected at a range between 50 ng and 15 ng of the patulin producing strain *P. griseofulvum* CBS 110.420 DNA in the presence of 50 ng of DNA from a non-producing strain (Fig. 2b).

3.4. Sensitivity of PCR for detecting patulin producing moulds on artificially inoculated food matrices

The sensitivity of the PCR protocol developed with the primer pair FC2/IDH2 was also evaluated analyzing the effects of different artificially inoculated foodstuff, including cooked products (mortadella, turkey breast, and ham), ripened foods (dry-cured ham, dry-fermented sausage "salchichón", and ripened cheese), and fruits (pear, apple, and peach). In all the artificially inoculated food matrices the expected 496-bp-amplicon was detected.

The lower levels of inoculated conidia yielding 496-bp-amplicon in the food products ranged from approximately 2.2×10^2 to 2.7×10^3 conidia g^{-1} in cooked products, 1.8×10^2 to 4.8×10^2 conidia g^{-1} in ripened food, and from 1.9×10^3 to 2.2×10^3 conidia g^{-1} in fruits (Table 3).

The occurrence of PCR inhibitors deriving from the assayed foods was evaluated by comparing the amplification of DNA from pure cultures of patulin producing mould in the presence of non-inoculated food-derived DNA and DNA from pure cultures. No inhibition of PCR due to cooked turkey breast and dry-cured ham components was found, since amplification was detected in all the assays (Fig. 3a and b). Amplification was obtained from each reaction in fruits, except when 5 μL of DNA from non-inoculated samples were added (Fig. 3c). False positive results were not

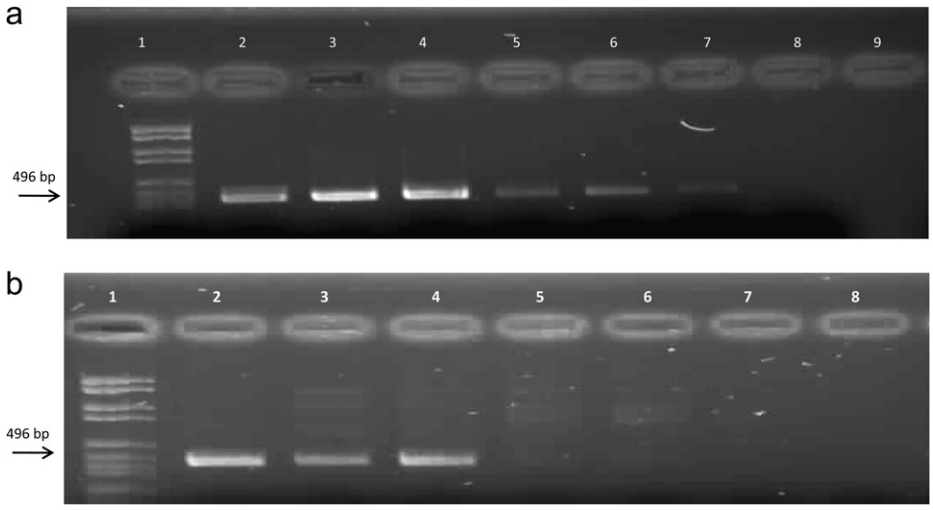

Fig. 2. (a) Agarose gel electrophoresis of PCR sensitivity assay with primer pair FC2/IDH2 using 50, 25, 15, 5, 2.5, 0.5, and 0.25 ng of *Penicillium expansum* CECT 2278 DNA (lanes 2, 3, 4, 5, 6, 7, and 8). Lane 1: DNA molecular size marker of 2.1–0.15 kbp. Lane 9: negative control using DNA from non-producing *Penicillium aurantiogriseum* CECT 2320. (b) Agarose gel electrophoresis of PCR sensitivity assay with primer pair FC2/IDH2 using 50 ng of the non-patulin producing *Penicillium nordicum* CBS 101.769 DNA mixed with 50, 25, 15, 5, 2.5, and 0.5 ng (lanes 2, 3, 4, 5, 6, and 7) of *Penicillium griseofulvum* CBS 110.420 DNA. Lane 1: DNA molecular size marker of 2.1–0.15 kbp. Lane 8: negative control using DNA from non-producing *Penicillium aurantiogriseum* CECT 2320.

Table 3
Detection limits (conidia g^{-1}) of PCR method with primer pair FC2/IDH2 using DNA from food products artificially inoculated reaching levels ranging from 10 to 10^6 conidia g^{-1} of patulin producing moulds.

Cooked products[a]			Cured products[b]			Fruits[c]		
Mortadella	Turkey breast	Ham	Dry-cured ham	Dry-fermented sausage "Salchichón"	Cured cheese	Apple	Peach	Pear
$(2.2 \pm 0.48)^{d} \times 10^2$	$(2.7 \pm 0.6) \times 10^3$	$(2.5 \pm 0.27) \times 10^2$	$(4.8 \pm 3.5) \times 10^2$	$(4.8 \pm 3.5) \times 10^2$	$(1.8 \pm 0.45)^{e} \times 10^2$ $(2.2 \pm 0.63)^{f} \times 10^2$	$(2.2 \pm 0.38) \times 10^3$	$(2.1 \pm 0.26) \times 10^3$	$(1.9 \pm 0.26) \times 10^3$

[a] Detection limits obtained for mortadella, turkey breast, and ham inoculated with *Penicillium commune* CBS 311.48.
[b] Detection limits obtained for dry-fermented sausage "salchichón" and dry-cured ham inoculated with *Penicillium griseofulvum* CBS 110.420 and for cured cheese inoculated with *Penicillium camemberti* CECT 2267 and *P. commune* CBS 341.59.
[c] Detection limits obtained for apple and pear inoculated with *Penicillium expansum* CECT 2280 and for peach inoculated with *P. griseofulvum* CBS 110.420.
[d] Data represent the means ± standards deviation SD of 3 independent assays.
[e] Detection limit obtained for cured cheese inoculated with *P. camemberti* CECT 2267.
[f] Detection limit obtained for cured cheese inoculated with *P. commune* CBS 341.59.

found when pure DNA alone from each non-inoculated food product was tested.

4. Discussion

To develop the present work the confirmation of which mould strains were patulin- and non-patulin producing was essential. All the patulin producing mould strains used were confirmed by MECE and/or HPLC-MS (Table 1). Both methods have been reported as sensitive to detect the production of patulin (Al-Hazmi, 2010; Gaspar & Lucena, 2009; Jalali, Khorasgani, Goudarzi, & Khoslesan, 2010; Murillo-Arbizu, González-Peñas, & Amézqueta, 2010; Sosa et al., 2002). All the strains generating patulin were considered as patulin producing strains to test the developed PCR method.

From the primers assayed in the development of the PCR protocol, only those derived from the *idh* gene showed specific amplification in all the patulin producing strains. Thus, with the selected primer pair FC2/IDH2, a specific 496-bp-amplicon was detected in all the producing strains identified by MECE and/or HPLC-MS. Although the *idh* gene has been used to design primers for PCR methods, it has only been usually applied to some specific species of *Penicillium* (Paterson, 2004, 2007) or *Aspergillus* (Varga et al., 2003). However, in the present work, patulin producing strains from three different genera were detected.

As regards sensitivity, the method was able to detect down to 0.5 ng of DNA from patulin producing moulds. These results proved to be more precise than those reported in other methods developed to detect different toxigenic moulds, e.g., aflatoxin producing ones (Färber, Geisen, & Holzapfel, 1997).

In addition, the presence of non-patulin producing mould DNA in PCR did not interfere substantially in the detection of small amounts of patulin producing mould DNA. Nevertheless the degree of sensitivity measured in this assay was slightly lower, since the minimal DNA concentration of a producing strain found was 15 ng instead of 0.5 ng (detected when only DNA of producing mould was assayed). This effect on the level of PCR sensitivity could be justified by the inhibitory action of the large amount of unspecific DNA over low concentrations of specific DNA, apparently by competition (Mayer, Bagnara, Färber, & Geisen, 2003).

PCR could detect patulin producing strains at levels as low as 1.8×10^2 or 2.7×10^3 conidia g^{-1}, depending on the type of tested food matrices. The optimal sensitivity level of the method could be actually validated by the fact that no inhibition was observed under the influence of the tested food matrices components in the inhibition assays. The only exception was the case of fruit samples, for which inhibition was detected only when a big quantity of food DNA was assayed. These findings agree with results involving the lowest detection limit (from 1.9×10^3 to 2.2×10^3 conidia g^{-1}) obtained in the artificially inoculated food matrix, in contrast with the remaining tested foods. Polysaccharides (Fang, Hammar, & Rebecca, 1992; Murray & Thompson, 1980) and polyphenolic compounds (Couch & Fritz, 1990; Katterman & Shattuck, 1983) might cause difficulties in DNA extraction from this food group and in the subsequent PCR amplification. Therefore, in order to improve PCR sensitivity with fruit samples, a short period of enrichment (from 12 to 48 h) might be suggested. Despite the lower sensitivity in fruits, the developed method had an accurate detection limit. In fact, the limits achieved were higher than those reported by Färber et al. (1997) and than the values given by Shapira et al. (1996), who applied an enrichment period of 24 h previous to PCR, and detected 10^2 conidia g^{-1}.

The method described also demonstrated a reasonably efficient speed, as it enabled the detection of patulin producing strains in about 8 h (5 h for treatment and DNA extraction from food, and 3 h for analyzing and visualizing PCR results). This method proved to run then at a faster rate than traditional culture methods, which need

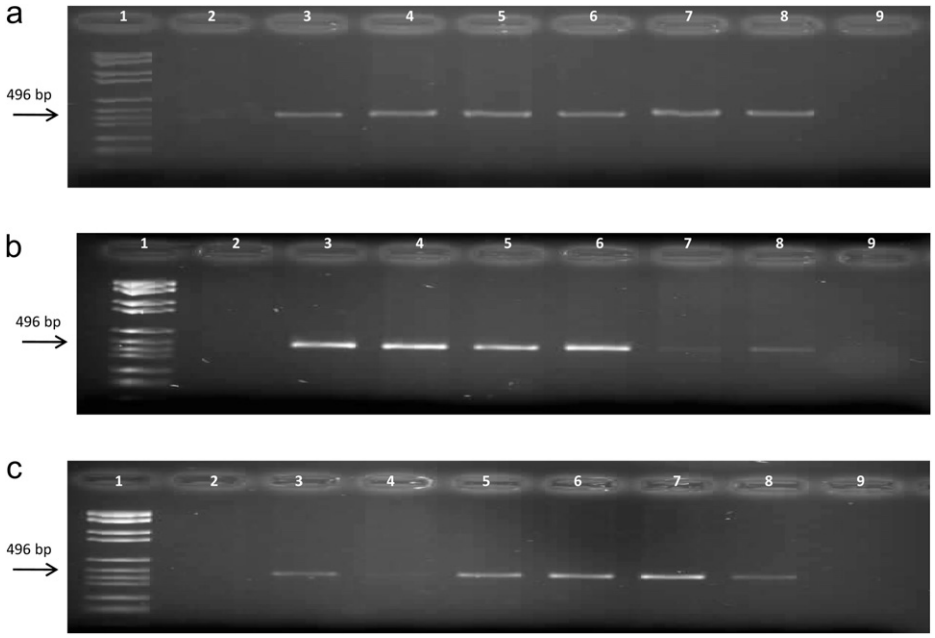

Fig. 3. Agarose gel electrophoresis of PCR products obtained using primer pair FC2/IDH2 in inhibition assays with food matrices: cooked turkey breast (a), dry-cured ham (b), and apple (c). Lane 1: molecular weight marker of 2.1 to 0.15 kbp. Lane 2: 5 μL of pure DNA of the non-inoculated assayed food. Lane 3: 5 μL of DNA from pure culture of patulin producing strains (*Penicillium commune* CBS 311.48 (a), *Penicillium griseofulvum* CBS 110.420 (b), and *Penicillium expansum* CECT 2280 (c)). Lanes 4, 5, 6, 7, and 8: 5 μL of pure DNA from patulin producing strains mixed with 1, 2, 3, 4, and 5 μL of pure DNA from the assayed food. Lane 9: negative control using sterile deionized water.

the identification of mould strains and then culture for patulin production analysis, requiring overall more than one week (Dombrink-Kurtzman & Blackburn, 2005; Erdogan et al., 2003).

The PCR method thus achieved a good deal of success in this study because of its capability to be used early and directly for the detection of patulin producing moulds in food. The method also may allow the location of low mould contamination, a more than likely agent for health risk, problems, and any other related consumer issues.

The legislation currently in force mainly regulates the maximum patulin levels in products derived from apples and also in baby food, ranging from 10 to 50 μg kg^{-1} (Commission Regulation (EC) No 1881/2006). Nevertheless, the current norms did not provide any regulation in terms of the maximum levels allowed for toxigenic moulds in food; neither does the system work effectively or fast enough to achieve this objective. PCR, as tested in this work, may be used as a routine method to detect patulin producing moulds in HACCP systems in the food industry, in comparison with new PCR techniques, such as Real Time PCR which requires expensive equipments and consumables as well as qualified staff. In the HACCP programs, the PCR sensitivity levels achieved could allow the monitoring of the target moulds so that the appropriate corrective actions may be taken (e.g., to prevent fungal growth and minimize the hazard derived from patulin formation). The effective detection of patulin producing moulds would allow the classification of ingredients for either their immediate processing or for convenient storage.

5. Conclusion

The PCR protocol developed in this study demonstrated that patulin producing mould strains could be effectively detected. This detection was based on the selective amplification of the *idh* sequence, denoting high sensitivity and specificity.

Besides the PCR-based method showed high sensitivity in the detection of producing strains in food samples and it could be proposed for routine analysis in HACCP systems in the food industry.

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