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

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ABSTRACT

Ochratoxin A (OTA) is a mycotoxin produced by several *Penicillium* and *Aspergillus* species growing in food commodities. To prevent OTA in foods it is necessary to have rapid and specific methods for early detection of producing moulds regardless of species and genera. In this work a PCR method to detect ochratoxigenic moulds has been developed. For this purpose, 75 mould strains belonging to species usually reported in food products were used. Their OTA production was checked by micellar electrokinetic capillary electrophoresis (MECE) and high-pressure liquid chromatography-mass spectrometry (HPLC-MS). A specific amplicon of 459 bp was detected by using the designed PCR protocol only in the OTA producing strains. The detection limit of the developed PCR protocol was estimated for 25 pg of mould DNA from pure cultures and from about 10^2–10^4 cfu/g when it was evaluated directly on artificially inoculated food. Its functionality in naturally infected samples was also demonstrated. In conclusion, the developed PCR method could be used for detecting ochratoxigenic moulds in foods and consequently for monitoring these moulds in the HACCP programs.

1. Introduction

Moulds represent a potential human hazard because of their ability to synthesize mycotoxins which can be produced in foods when environmental conditions are appropriate. Ochratoxin A (OTA), one of these toxic secondary metabolites, has nephrotoxic, inmunotoxic, hepatotoxic, genotoxic, and teratogenic effects (Bacha, Atoui, Mathieu, Liboz, & Lebrihi, 2009; Pfohl-Leszkowicz & Manderville, 2007). OTA has been also considered as a possible human carcinogen included in the group 2B (International Agency for Research on Cancer, 1993; Petzinger & Ziegler, 2000).

OTA occurs naturally due to fungal contamination in a variety of foods and beverages including fruits (Marino, Nostro, & Fiorentino, 2009), coffee (Batista, Chalfoun, Prado, Schwan, & Wheals, 2003), cereals (Juan, Moltó, Lino, & Mañes, 2008), cured meat products (Iacumin et al., 2009; Toscani et al., 2007), cheeses (Cabañes, Bragulat, & Castellá, 2010), spices (Castellá et al., 2002), and nuts (Marín, Hodžić, Ramos, & Sanchís, 2008).

Several *Aspergillus* and *Penicillium* species have been reported as ochratoxigenic moulds. With regard to *Aspergillus* species, *Aspergillus ochraceus*, *Aspergillus niger*, *Aspergillus carbonarius*, *Aspergillus westerdijikiae*, and *Aspergillus steynii* are important OTA producers responsible for the contamination of products such as coffee, raisins, grape juice, spices, or wines (Bacha et al., 2009; Gil-Serna, Vázquez, Sardiñas, González-Jaén, & Patiño, 2011). Regarding to *Penicillium* species, *Penicillium verrucosum* and *Penicillium nordicum*, isolated mainly from cereals and derived products (Cabañes, Bragulat, Abarca, Castellá, & Cabañes, 2008), and fermented meats and cheeses (Castellá et al., 2002), respectively, have been also reported as OTA producers (Frisvad, Smedsgaard, Larsen, & Samson, 2004).

Inhibition of the growth of ochratoxigenic moulds in foods is the main strategy that the food industry can use to prevent the OTA presence in their products, since removing the mycotoxin from food is currently not feasible (Gil-Serna, Vázquez, Sardiñas, González-Jaén, & Patiño, 2009). To avoid contamination of food by ochratoxigenic moulds, appropriate hygienic actions should be taken. In addition, it is required a rapid and specific method for early detection of producing moulds (Dao, Mathieu, & Lebrihi, 2005). In spite of the fact that methods based on morphological features could be used to detect OTA producing moulds, they are time-consuming, have little sensitivity, and normally require mycological expertise (Dao et al., 2005). In addition, it is necessary a confirmation of mycotoxin production using analytical methods. As an alternative, DNA-based methods such as polymerase chain reaction (PCR) are usually rapid, sensitive, and specific, and they allow obtaining an accurate detection of mould species (Borman,

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Linton, Miles, & Johnson, 2008). Several PCR protocols based on genes involved in the mycotoxin biosynthesis pathway have been applied to detect toxigenic moulds commonly found in foods (Del Fiore et al., 2010; Paterson, 2006). Although the OTA biosynthesis pathway has not been completely elucidated (Dao et al., 2005; Färber & Geisen, 2004), different PCR protocols to detect specific ochratoxigenic mould species have been developed. They have been mainly based on the OTA *polyketide synthase* (*otapks*PN) and the OTA *non ribosomal peptide synthetase* (*otanps*PN) genes (Bogs, Battilani, & Geisen, 2006), located in a partial gene cluster of the OTA biosynthetic genes (Karolewiez & Geisen, 2005). However from the point of view of the food safety, it would be of great interest to develop a PCR method able to detect OTA producing moulds regardless of their genus and species. For this purpose, the *otanps*PN and o*tapks*PN genes could be evaluated.

On the other hand, the potential application of the PCR protocol to detect OTA producers should be evaluated directly on food commodities. However, to develop a PCR method capable of detecting ochratoxigenic moulds in food samples it should be considered that food components, such as fat, polysaccharides, polyphenols, and other compounds may inhibit an efficient amplification (Demeke & Jenkins, 2010).

The main objective of this study was to develop a PCR method to detect ochratoxigenic mould strains regardless of genus and species in food products.

2. Material and methods

2.1. Mould strains

Seventy five mould strains belonging to species commonly found on foods were used in this study (Table 1). They were obtained from different Culture Collections: the Spanish Type Culture Collection (CECT, Spain), the Centraalbureau voor Schimmelcultures (CBS, The Netherlands), the Type Culture Collection of the Department of Biotechnology from the Technical University of Denmark (IBT, Denmark), the Food Hygiene and Safety Collection of the University of Extremadura (FHSC, Spain), and three strains kindly supplied by Dr. Covadonga Vázquez (University Complutense of Madrid, Spain).

2.2. Evaluation of OTA production

Production of OTA was determined after growing each mould strain in Potato Dextrose Agar (PDA, Scharlau Chemie S.A., Barcelona, Spain) and incubating for 15 days at 25 °C. The content of three petri dishes was extracted with 150 mL of chloroform, filtered twice through anhydrous sodium sulphate, and evaporated in a rotatory evaporator at 40 °C as described by Núñez, Westphal, Bermúdez, and Asensio (2007). The residue was resuspended in 5 mL of chloroform, filtered through a 0.45-μm pore size nylon membrane (Micron Separation Inc., Westborough, Massachusetts, USA), and evaporated to dryness under a gentle stream of nitrogen. The extracts were stored at 4 °C in the dark until required and resuspended in 200 μL of acetonitrile just before analysis. OTA production was analysed by micellar electrokinetic capillary electrophoresis (MECE) and by high-pressure liquid chromatography-mass spectrometry (HPLC-MS).

MECE analysis was carried out according to Martín, Jurado, Rodríguez, Núñez, and Córdoba (2004) in a Beckman P/ACE 5500 system model with a photodiode array detector (Beckman Instruments, Inc., Fullerton, California, USA). A fused silica capillary of 57 cm length and 75 mm inside diameter was used for separation with 25 mM sodium tetraborate and 50 mM SDS (pH 9) as running buffer, at 15 kV, maximum current at 200 mA, and a capillary cassette temperature of 23 °C. The absorbance was recorded at 200 and 280 nm wavelengths. For each peak, a spectrum of absorbance between 190 and 600 nm was obtained in the photodiode array detector.

HPLC-MS analysis was performed according to Núñez et al. (2007) in a Hewlett Packard series 1100 apparatus (Hewlett Packard, Palo Alto, California, USA). A Supelcosil LC-18 column (SUPELCO, Bellefonte, Pennsylvania, USA) was used with mobile phases (A) 100% water and (B) 0.05% trifluoroacetic acid in acetonitrile in a gradient from 10% to 99% B. OTA was identified in a Finnigan LCQ Mass Spectrometer (Finnigan, San Jose, California, USA) with atmospheric pressure chemical ionization source (APCI), according to their retention time and molecular mass.

In both MECE and HPLC-MS analyses, OTA (Sigma Aldrich Co., St. Louis, Missouri, USA) was used as standard.

2.3. DNA isolation from mould strains

Every mould strain was 3-point inoculated on PDA and incubated for 4 days at 25 °C. Grown mycelium was then scraped off the agar and about 50 mg of isolated mycelium from each strain were used for genomic DNA extraction following the method described by Sánchez, Rodríguez, Casado, Martín, and Córdoba (2008). This DNA extraction method included a digestion with proteinase K (1 mg/mL, Sigma Aldrich Co.) and lyticase (4 U/mL, Sigma Aldrich Co.) prior to using a mortar and pestle grinding combined with the semiautomatic vacuum system ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, California, USA).

Concentration of DNA was determined in 1% agarose gels by submerged electrophoresis using 1XTAE buffer at 90 V for 15 min. Gels were stained with ethidium bromide (0.5 μg/mL, Sigma Aldrich Co.) and DNA was visualized by using UV transillumination G127 Box of Syngene (Synoptics group, Frederick, Maryland, USA). Besides they were photographed using the integrated camera and the software GeneSnap of Syngene.

2.4. PCR assays

Genomic DNA from 11 OTA producing strains (*Aspergillus awamori* CBS 101702, *Aspergillus foetidus* CBS 101708, *A. niger* CECT 20157, *A. ochraceus* CBS 589.68, *Aspergillus tubingensis* CECT 20543 and CECT 20545, *P. nordicum* CBS 101769, *P. verrucosum* FHSC-3, CECT 2906, and CBS 323.92, and *Penicillium viridicatum* CECT 2320) was amplified using three primer pairs and three conventional PCR methods previously reported by Dao et al. (2005) and Bogs et al. (2006) (Table 2). Both primer pairs AoLC35F/AoLC35R and otapksF/otapksR were based on the *otapks*PN gene, while the primer pair otanpsF/otanpsR on the *otanps*PN gene. DNA from the non producing strain *Aspergillus versicolor* CECT 2664 was used as negative control. The primers showing the most specific and intense amplification of OTA producing moulds were selected for further analysis.

PCR products obtained with the selected primer pair (otanpsF/otanpsR) were purified using the MinElute® PCR Purification Kit, following the manufacturer's instructions (QIAGEN, Hilden, Germany) and sequenced at Instituto de Biomedicina (CSIC, Valencia, Spain). To test the specificity of the obtained sequences, they were compared with the *otanps*PN gene sequence published in the NCBI database (GenBank accession number AY557343) (www.ncbi.blast.com). These sequences were then analysed and aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to design three new primer pairs from conserved regions by means of the Primer Express software (Applied Biosystems) (Table 2). These primers were tested with the 11 OTA producing strains above mentioned in order to select the most specific primer pair.

Table 1
Results of the ochratoxin A (OTA) production and the PCR reactions with primer pairs F1OT/R1OT and Bt2a/Bt2b from the reference mould strains used in this study.

Species designation	Strain reference	[f]Information provided by culture collections	[g]OTA production (μg/L) as detected by MECE/HPLC-MS	PCR results	
				[h]F1OT/R1OT	[i]Bt2a/Bt2b
Aspergillus awamori	[a]CBS 101702	+	91.3	+	+
Aspergillus carbonarius	CECT 20384	U	37.2	+	+
Aspergillus carbonarius	CECT 2086	U	71.1	+	+
Aspergillus flavus	[b]CECT 2684	U	–	–	+
Aspergillus flavus	CECT 2687	U	–	–	+
Aspergillus flavus	[c]IBT 3696	U	–	–	+
Aspergillus flavus	CBS 573.65	U	–	–	+
Aspergillus flavus	CBS 120.62	U	–	–	+
Aspergillus foetidus	CBS 101708	+	99.7	+	+
Aspergillus fumigatus	CBS 192.65	U	–	–	+
Aspergillus niger	CECT 20157	+	93.4	+	+
Aspergillus niger	CECT 2088	U	65.3	+	+
Aspergillus niger	[d] FHSC-42	+	70.4	+	+
Aspergillus ochraceoroseus	CBS 101887	U	–	–	+
Aspergillus ochraceus	CBS 589.68	+	95.7	+	+
Aspergillus ochraceus	CECT 2092	U	69.9	+	+
Aspergillus ochraceus	CECT 2093	U	70.4	+	+
Aspergillus oryzae	CECT 2095	U	–	–	+
Aspergillus oryzae	CECT 20168	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 steynii	CBS 112813	–	–	–	+
Aspergillus tamarii	CBS 575.65	U	–	–	+
Aspergillus tamarii	CBS 109.63	U	71.4	+	+
Aspergillus terreus	CBS 601.65	U	–	–	+
Aspergillus toxicarius	CBS 822.72	U	–	–	+
Aspergillus tubingensis	CECT 20543	+	99.8	+	+
Aspergillus tubingensis	CECT 20545	+	99.9	+	+
Aspergillus versicolor	CECT 2664	U	–	–	+
Aspergillus versicolor	CECT 2903	U	–	–	+
Aspergillus versicolor	CECT 2814	U	–	–	+
Aspergillus westerdijkiae	CECT 2948	U	34.7	+	+
Aspergillus westerdijkiae	[e]AOPD16	+	26.6	+	+
Aspergillus westerdijkiae	[e]PDF1	+	99.8	+	+
Aspergillus westerdijkiae	[e]3.58	+	99.7	+	+
Emericella heterothallica	CBS 488.65	U	–	–	+
Emericella nidulans	CBS 465.65	U	1.1	+	+
Emericella rugulosa	CBS 133.60	U	–	–	+
Emericella variecolor	CBS 133.55	U	27.0	+	+
Penicillium aurantiogriseum	CECT 2918	U	–	–	+
Penicillium aurantiogriseum	CBS 112021	U	–	–	+
Penicillium aurantiogriseum	CECT 2264	U	28.3	+	+
Penicillium camemberti	CECT 2267	U	–	–	+
Penicillium camemberti	FHSC-1	U	–	–	+
Penicillium camemberti	CBS 273.97	U	–	–	+
Penicillium carneum	CBS 468.95	U	32.7	+	+
Penicillium commune	CBS 311.48	U	–	–	+
Penicillium commune	CBS 341.59	U	–	–	+
Penicillium commune	CBS 247.32	U	–	–	+
Penicillium dipodomyicola	IBT 26223	U	–	–	+
Penicillium dipodomyicola	CBS 110425	U	–	–	+
Penicillium dipodomyicola	CBS 110426	U	–	–	+
Penicillium expansum	CECT 2278	U	–	–	+
Penicillium expansum	CECT 20140	U	–	–	+
Penicillium expansum	CECT 2279	U	–	–	+
Penicillium expansum	CECT 2280	U	–	–	+
Penicillium griseofulvum	IBT 14319	U	–	–	+
Penicillium griseofulvum	CBS 485.84	U	–	–	+
Penicillium griseofulvum	CBS 110420	U	–	–	+
Penicillium griseofulvum	CECT 2919	U	–	–	+
Penicillium melanoconidium	CBS 64195	U	–	–	+
Penicillium melanoconidium	CBS 109605	U	36.7	+	+
Penicillium nordicum	CBS 110769	+	99.4	+	+
Penicillium polonicum	FHSC-2	U	–	–	+
Penicillium polonicum	CBS 112490	U	–	–	+
Penicillium polonicum	CBS 639.95	U	–	–	+
Penicillium polonicum	CBS 101479	U	–	–	+
Penicillium verrucosum	FHSC-3	+	97.6	+	+
Penicillium verrucosum	CECT 2906	+	99.6	+	+
Penicillium verrucosum	CBS 323.92	+	99.0	+	+

Table 1 (*continued*)

Species designation	Strain reference	[f]Information provided by culture collections	[g]OTA production (μg/L) as detected by MECE/HPLC-MS	PCR results	
				[h]F1OT/R1OT	[i]Bt2a/Bt2b
Penicillium viridicatum	CECT 2320	+	95.3	+	+
Rhizopus oryzae	CBS 607.68	U	−	−	+

All OTA producing mould strains are shaded.
[a] CBS: Centraalbureau voor Schimmelcultures in The Netherlands.
[b] CECT: Spanish Type Culture Collection.
[c] IBT: Type Culture Collection of the Department of Biotechnology from the Technical University of Denmark.
[d] FHSC: Food Hygiene and Safety Collection of the University of Extremadura (Spain).
[e] Strain kindly supplied by Dr. Covadonga Vázquez (University Complutense of Madrid, Spain).
[f] (+): ability to produce OTA based on information available in the culture collections; (U): information about OTA production is unavailable in the culture collections.
[g] positive (+) and negative (−) represent detected and non detected OTA production determined by MECE and HPLC-MS, according to detection by both methods or one of them.
[h] positive (+) and negative (−) represent detected and non detected PCR product of 459 bp, respectively.
[i] positive (+) represents detected PCR product of 453 bp.

After optimizing the PCR conditions, amplification reactions were carried out in a final volume of 50 μL, containing 5 μL of 10X Mg free PCR buffer, 2 μL of $MgCl_2$ (50 mM), 1 μL of deoxynucleotides (dNTPs) mix (10 mM), 2 μL of each primer (10 mM), 0.5 μL of *Taq* polymerase (2 U/μL, Finnzymes, Espoo, Finland), and 5 μL of genomic DNA (10 ng). The amplification conditions were: initial denaturation step at 94 °C for 5 min; 33 cycles at a melting temperature of 95 °C for 30 s, annealing temperature of 57 °C for 40 s, and extension temperature of 72 °C for 1 min. A final extension of 5 min at 72 °C was also included. A negative control using DNA from the OTA non producing *A. versicolor* CECT 2664 was included in each PCR assay.

Amplification products were analysed in 2% agarose gels using 1XTAE buffer at 80 V for 1 h. Gels were stained with ethidium bromide, and the products visualized and photographed as described above. A DNA molecular size marker of 2.1–0.15 kbp (Roche Diagnostics, Indianapolis, Indiana, USA) was used to determine the size of the PCR products.

The specificity of the PCR protocol for the detection of OTA producing moulds was assayed in triplicate with the 75 reference mould strains.

In addition, the presence of mould DNA was tested by a PCR protocol designed on the universal fungal *β-tubulin* gene with primers Bt2a and Bt2b (Glass & Donaldson, 1995). The amplification program was: 1 cycle of 5 min at 94 °C, 32 cycles of 1 min at 94 °C, 1 min at 68 °C and 1 min at 72 °C and finally 1 cycle of 5 min at 72 °C.

2.5. Sensitivity of the PCR assay with pure cultures

To evaluate the sensitivity of the designed PCR method, several concentrations of DNA from the OTA producing strain *P. verrucosum* CECT 2906, ranging from 50 ng to 0.05 pg, were assayed using the primer pair F1OT/R1OT. The detection limit of the PCR method was defined as the lowest DNA concentration of *P. verrucosum* CECT 2906 detected in all reactions.

The sensitivity of the developed method was also tested in presence of high amount of DNA from an OTA non producing strain. Thus, 50 ng of DNA from *Penicillium aurantiogriseum* CECT 2918 were mixed with different amounts of DNA (ranging from 5 ng to 15 pg) from the producing strain *P. viridicatum* CECT 2320.

2.6. Sensitivity of PCR on artificially inoculated food matrices

The sensitivity of the PCR method was also assayed with DNA extracted from 3 non-sterile food groups (cooked, ripened, and miscellaneous products) inoculated with conidia of different OTA producing strains. Cooked turkey breast, cooked ham, and mortadella were thus inoculated with *P. verrucosum* FHSC-3, and dry-cured ham, dry-fermented sausage "salchichón", and ripened cheese were inoculated with *P. nordicum* CBS 110769. Finally, a miscellaneous group consisting of fruits (grape and plum), spices (paprika), and nuts (almond and walnut) was inoculated with *A. ochraceus* CBS 589.68. Selection of mould strains used for inoculating foods was based on the most common species found in each type of the tested products.

The conidia of each mould strain used for inoculation were extracted by flooding three 20-day-old plates of PDA with 5 mL of sterile deionized water containing 10% (v/v) glycerol and rubbing the surface with a glass rod. The concentration of conidia suspensions was quantified by microscopy, using a Neubauer counting chamber. Two hundred μL of decimal dilutions of conidia were used

Table 2
Sequences of primers used in this study and PCR product sizes.

Primer pairs	Sequence nucleotides (5′-3′)	GenBank accession number/Positions	Expected PCR product (bp)	References
AoLC35F	GCCAGACCATCGACACTGCATGCTC	[a]AY583208	520	Dao et al., 2005
AoLC35R	CGACTGGCGTTCCAGTACCATGAGCC			
otapksF	TACGGCCATCTTGAGCAACGGCACTGC	[b]AY196315	500	Bogs et al., 2006
otapksR	ATGCCTTTCTGGGTCAGTA			
otanpsF	AGTCTTCGCTGGGTGCTTCC	[c]AY534879	750	Bogs et al., 2006
otanpsR	CAGCACTTTTCCCTCCATCTATCC			
F2OT	GTGACTGGGTTGAACTTCTCGCC	[d]4804	373	This study
R2OT	GGCGGTGGACCCCTCTCC	5177		
F3OT	CGGCCAGACAATGTCAATCAAG	4653	509	This study
R4OT	TCTCCCGAGGTCGGCCGC	5162		
F1OT	GCCCAACGACAACCGCT	4748	459	This study
R1OT	GCCATCTCCAAACTCAAGCGTG	5207		

[a] Published sequence of the ochratoxin A *polyketide synthase (otapks*PN*)* gene of *Aspergillus ochraceus*.
[b] Published sequence of the *otapks*PN gene of *Penicillium nordicum*.
[c] Published sequence of the ochratoxin A *non ribosomal peptide synthetase* (*otanps*PN) gene of *P. nordicum*.
[d] Positions are in accordance with the published sequences of the *otanps*PN gene of *P. nordicum* (GenBank accession number AY557343).

to inoculate 5 g of each food product to obtain final inoculation levels ranging from 10^6 to 10 conidia/g. Ten mL of Tris–HCl buffer (pH 8.0) were then added to the inoculated food samples and the resulting mixture was homogenized in a filter bag BagPage (Interscience, Paris, France) using a stomacher machine (IUL Instruments, Barcelona, Spain). In order to directly estimate the load of the inoculated moulds, 10-fold dilutions from the obtained filtrate solutions were prepared in 0.1% sterile peptone water. A volume of 0.1 mL of these solutions was plated out on PDA and incubated at 25 °C for 4 days to calculate the number of colony forming units (cfu) per gram of the corresponding foods.

The natural mould contamination of the samples was lower than 10 cfu/g. When its morphological characterization was performed no typical colonies of *P. verrucosum, P. nordicum*, or *A. ochraceus* were found.

For DNA extraction, the remaining volume of each filtrate was transferred to a sterile tube, and centrifuged at 2500 rpm for 30 s, then at 13,000 rpm for 10 min. The supernatants were removed and the pellets were resuspended in 100 μL of sterile deionized water previously heated and boiled at 95 °C for 10 min. After cooling this solution on ice for 10 min, 200 μL cetyltrimethylammonium bromide (CTAB) extraction buffer (20 g/L CTAB, 1.4 M NaCl, 20 mM Na_2EDTA, 0.1 M Tris–HCl, pH 8.0), 400 μL 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), and 200 μL of lyticase (4 U/mL) were added and incubated at 65 °C for 1 h. Samples were then washed, resuspended in 600 μL chloroform, and centrifuged at 13,000 rpm for 20 min. After adding 20 μL of RNase A solution (10 mg/mL, Sigma Aldrich Co.) to the obtained supernatant, it was incubated at 37 °C for 1 h. A volume of 600 μL chloroform was then added. The resulting mixture was vortexed and centrifuged at 13,000 rpm for 5 min. Finally, the aqueous phase was processed with the EZNA Fungal DNA Mini Kit (Omega bio-tek, Doraville, USA), starting with DNA precipitation by adding 600 μL of cold isopropanol. In the final step, DNA was eluted in 25 μL of elution buffer pre-incubated at 65 °C and kept at −20 °C until using as template for PCR. Negative controls from non inoculated food samples were also carried out following the protocol described above. All these assays were carried out in triplicate.

Three replicates of 5 μL DNA extracted from each inoculated food were assayed per amplification reaction. The non inoculated negative controls were also performed in triplicate. PCR results were then visualized on 2% agarose gels as described in Section 2.4.

The detection limit of the PCR method in the above food samples was estimated as the lowest count of the OTA producing moulds in each tested food showing the specific amplicon of 459 bp. In addition, the presence of mould DNA was tested in all samples by PCR based on the *β-tubulin* gene following the procedure indicated in Section 2.4. To analyse the influence of food components on the DNA extraction of moulds and the subsequent PCR reaction, 5 μL of pure DNA from OTA producing *P. verrucosum* FHSC-3, *P. nordicum* CBS 110769, and *A. ochraceus* CBS 589.68 were mixed with different volumes (1, 2, 3, 4, and 5 μL) of DNA obtained from each corresponding non inoculated food. The products resulting from the amplification reactions were compared with those obtained by amplifying pure DNA from the ochratoxigenic mould strains.

2.7. Validation of PCR in naturally infected food samples

The ability of the PCR method to detect OTA producing moulds in naturally infected samples was assayed with DNA extracted from 15 dry-cured ham, 15 paprika, and 15 durum wheat samples. DNA extraction was carried out following the method described in Section 2.6. DNA was then subjected to the PCR method designed as described in Section 2.4. Furthermore, the presence of fungal DNA was analysed by PCR based on the *β-tubulin* gene as described in Section 2.4.

2.8. Statistical analysis

All the statistical analyses were performed with the SPSS v.15.0. One way analysis of variance (ANOVA) was carried out to determinate significant differences within and between groups. Tukey's test was applied to compare the mean values. Statistical significance was set at $P \leq 0.05$.

3. Results

3.1. Evaluation of OTA production by MECE and HPLC-MS

MECE and HPLC-MS analyses confirmed OTA production in all 15 strains identified as OTA producers by their respective Culture Collections (Table 1). Furthermore, 12 strains with no information on OTA production were able to produce this mycotoxin on PDA (Table 1), while the remaining 48 strains did not show production of OTA by MECE or HPLC-MS (Table 1).

3.2. PCR assays for detecting OTA producing moulds

Several primer pairs previously designed from different genes involved in the OTA biosynthetic pathway were tested in the present study. With the primer pair AoLC35F/AoLC35R, the expected 520 bp amplicon was only obtained in 4 of the 11 tested strains (*P. viridicatum* CECT 2320, *P. verrucosum* FHSC-3, *A. tubingensis* CECT 20545, and *P. nordicum* CBS 101769). With primer pair otapksF/otapksR, most of the tested producing strains did not show amplification. Only for *A. tubingensis* CECT 20545 and *P. nordicum* CBS 101769 a very faint 500 bp amplification product was observed. The primer pair otanpsF/otanpsR generated a unique and intense amplicon of 750 bp in 6 of the 11 OTA producing strains tested. The non OTA producing *A. versicolor* CECT 2664 did not show the expected PCR products with none of the primer pairs (data not shown).

The amplicons obtained with the primer pair otanpsF/otanpsR were sequenced because of their higher specificity. When the obtained sequences were analysed, all of them showed upper than 99% of similarity with the *otanps*PN gene sequence published in the NCBI database (GenBank accession number AY557343). From these sequences, 3 new primer pairs based on the conserved regions of the *otanps*PN gene were then designed (Table 2). The PCR products expected with the primer pairs F2OT/R2OT and F3OT/R4OT were only visualized in 5 of the 11 tested OTA producing strains (Fig. 1a and b). However a single amplicon of the expected size (459 bp) was detected in all of the 11 tested OTA producing strains with primer pair F1OT/R1OT (Fig. 1c). PCR amplification was not detected in the non producing *A. versicolor* CECT 2664 with none of the designed primer pairs. From these results, the primer pair F1OT/R1OT was selected to be tested with the 75 mould strains used in this study. The 459 bp expected product was observed in the 27 OTA producing mould strains (Table 1). None of the non ochratoxigenic reference strains gave a positive result with this primer pair. In addition, all producing and non-producing reference mould strains showed an amplicon of approximately 453 bp by PCR using the primer pair Bt2a/Bt2b based on the *β-tubulin* gene (Table 1).

3.3. Sensitivity of the PCR protocol for detecting OTA producing moulds in pure cultures

The sensitivity of the PCR assay with primers F1OT and R1OT was analysed using dilutions of DNA from a pure culture of the ochratoxigenic *P. verrucosum* CECT 2906. The detection limit was

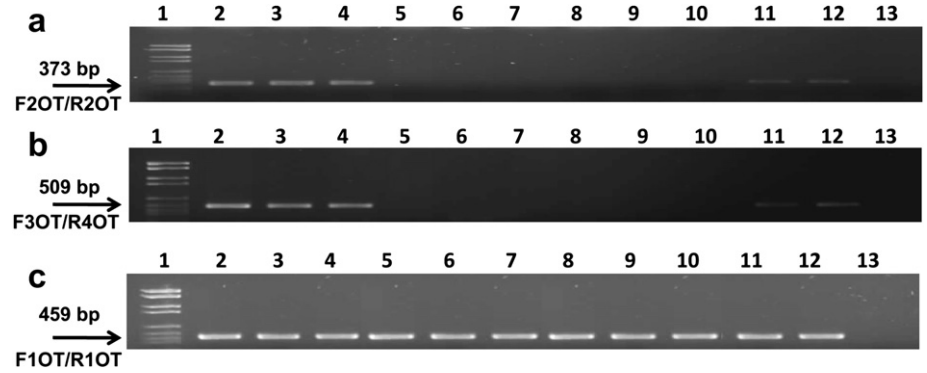

Fig. 1. Agarose gel electrophoresis of PCR products obtained with primer pairs F2OT/R2OT (a), F3OT/R4OT (b), and F1OT/R1OT (c). Lane 1: DNA molecular size marker of 2.1–0.15 kbp. Lane 2: *Penicillium viridicatum* CECT 2320. Lane 3: *Penicillium verrucosum* FHSC-3. Lane 4: *P. verrucosum* CECT 2906. Lane 5: *Aspergillus tubingensis* CECT 20543. Lane 6: *A. tubingensis* CECT 20545. Lane 7: *Aspergillus niger* CECT 20157. Lane 8: *Aspergillus awamori* CBS 101702. Lane 9: *Aspergillus foetidus* CBS 101708. Lane 10: *Aspergillus ochraceus* CBS 589.68. Lane 11: *P. verrucosum* CBS 323.92. Lane 12: *Penicillium nordicum* CBS 101769. Lane 13: Negative control using DNA from the non-producing strain *Aspergillus versicolor* CECT 2664.

25 pg of mould DNA (Fig. 2a). In addition, the 459 bp amplicon was detected using more than 50 pg DNA from the producing *P. viridicatum* CECT 2320 DNA in presence of 50 ng of DNA from a non producing strain (Fig. 2b).

3.4. Sensitivity of the PCR method for detecting OTA producing moulds on artificially inoculated food matrices

To demonstrate that the PCR is functional in a food matrix, different foods were inoculated with ochratoxigenic moulds. After performing the DNA extraction directly from the artificially contaminated samples and the optimized PCR with the primer pair F1OT/R1OT, the 459 bp amplification product was detected in all the food matrices as it was expected. The lowest concentration of conidia yielding the amplicon varied slightly between and within the food group. Thus the detection limit ranged from 2.1×10^3 to 2.8×10^3 cfu/g in cooked products, from 4.3×10^2 to 2.8×10^3 cfu/g in ripened foods, and from 2.6×10^2 to 2.6×10^4 cfu/g in the miscellaneous group (Table 3). However, for this parameter no significant differences ($P > 0.05$) between foods were found (Table 3). Furthermore the detection limit was reproducible in most of the food matrices assayed since low variations in the standard deviation were usually observed.

The presence of PCR inhibitors in the inoculated foods was evaluated by comparing the amplification of DNA from pure cultures of OTA producing moulds in presence of DNA from non inoculated foods with those amplicons obtained with DNA from pure cultures. Amplification was always obtained with the different tested volumes of DNA from cooked meat products, ripened food, and paprika, except when 5 μL of DNA from non inoculated samples were added (Fig. 3). When DNA from non inoculated fruits was used, PCR products were always detected, while no amplification was observed when adding 4 and 5 μL of DNA from non inoculated nuts. On the other hand, the amplicons showed some differences in their intensity in all food matrices but they were not related to the spiked amounts of non inoculated food DNA (Fig. 3). No false positive results were found when pure DNA from each non inoculated food product was only tested.

3.5. Ability of the PCR method for detecting OTA producing moulds on naturally infected food samples

The capacity of the designed PCR method for detecting ochratoxigenic moulds in naturally infected food samples was finally evaluated. Concretely, 15 dry-cured ham, 15 paprika, and 15 wheat semolina samples were analysed by the above described PCR method together with the PCR protocol based on the *β-tubulin* gene to assess the presence of fungal DNA.

The results confirmed the presence of mould DNA in all tested dry-cured ham samples by using the PCR based on the *β -tubulin* gene (Table 4). A total of 73% of these samples presented ochratoxigenic moulds when the designed PCR in this study was used (Table 4). Regarding to paprika and durum wheat semolina samples, 11 and 1 of them showed positive results, respectively, when using *β-tubulin* PCR, nevertheless none of them presented OTA producing moulds (Table 4).

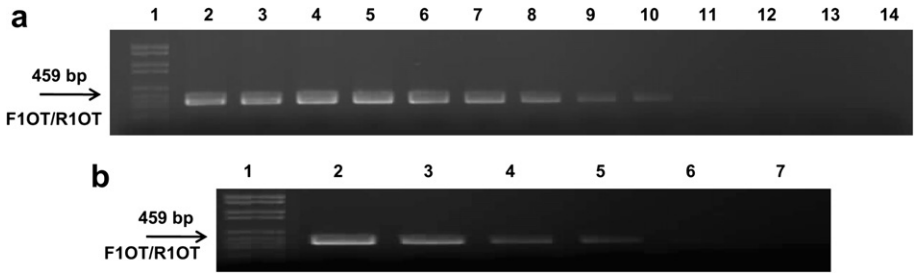

Fig. 2. (a) Agarose gel electrophoresis of PCR sensitivity assay with primer pair F1OT/R1OT using 50, 25, 15, 5, 2.5, 0.5, and 0.25 ng, 50, 25, 15, 5, and 0.05 pg of *Penicillium verrucosum* CECT 2906 DNA (lanes 2–13). Lane 1: DNA molecular size marker of 2.1–0.15 kbp. Lane 14: negative control using DNA from the non producing strain *Aspergillus versicolor* CECT 2664. (b) Agarose gel electrophoresis of PCR specificity assay using the primer pair F1OT/R1OT. Fifty ng of DNA from the non OTA producing *Penicillium aurantiogriseum* CECT 2918 were mixed with 5, 0.5 ng, and 50, 25, and 15 pg (lanes 2–6) of OTA producing *Penicillium viridicatum* CECT 2320 DNA. Lane 1: DNA molecular size marker of 2.1–0.15 kbp. Lane 7: Negative control using DNA from the non producing strain *A. versicolor* CECT 2664.

Table 3
Detection limits of the PCR method using primer pair F1OT/R1OT and DNA obtained from food products artificially inoculated with levels ranging from 10 to 10^6 conidia/g of ochratoxin A producing moulds.

Artificially contaminated food products	Inoculated mould strain	[a]Inoculum level (conidia/g)	[b]Detection limit (cfu/g)
Cooked products			
Cooked ham	*Penicillium verrucosum* FHSC-3	[c]$(2.2 \pm 1.15) \times 10^3$	$(2.5 \pm 0.31) \times 10^3$
Turkey breast		$(3.3 \pm 2.25) \times 10^3$	$(2.1 \pm 0.30) \times 10^3$
Mortadella		$(1.5 \pm 0.57) \times 10^3$	$(2.8 \pm 0.34) \times 10^3$
Cured products			
Dry-cured ham	*Penicillium nordicum* CBS 110769	$(2.9 \pm 1.91) \times 10^3$	$(2.8 \pm 0.30) \times 10^3$
Dry-fermented sausage "salchichón"		$(1.9 \pm 0.48) \times 10^3$	$(2.1 \pm 0.41) \times 10^3$
Cured cheese		$(2.1 \pm 0.15) \times 10^3$	$(4.3 \pm 1.52) \times 10^2$
Miscellaneous group			
Grape	*Aspergillus ochraceus* CBS 589.68	$(1.7 \pm 0.14) \times 10^2$	$(3.3 \pm 1.15) \times 10^2$
Plum		$(1.9 \pm 0.13) \times 10^2$	$(2.6 \pm 1.16) \times 10^2$
Paprika		(2.1 ± 0.11) x 10^3	(2.3 ± 0.57) x 10^3
Almond		$(1.8 \pm 0.34) \times 10^4$	(1.7 ± 0.58) x 10^4
Walnut		$(2.0 \pm 0.51) \times 10^4$	$(2.6 \pm 0.58) \times 10^4$

[a] Data represent the number of conidia per gram of food sample (conidia/g) quantified by microscopy using a Neubauer counting chamber.
[b] Data represent the colony forming units per gram of food sample (cfu/g) obtained after culturing each sample on Potato Dextrose Agar.
[c] Data represent the means ± the standard deviations of 3 independent assays.

4. Discussion

In the present study, a PCR method for the detection of OTA producing moulds was developed. The ability of producing OTA by 75 reference mould strains was firstly analysed by MECE and HPLC-MS. These analytical techniques have been reported as sensitive tools to detect production of OTA (Brera, Grossi, & Miraglia, 2005). A total of 27 mould strains from the three tested genera (*Aspergillus, Emericella, and Penicillium*) produced OTA on PDA when analysed by both analytical techniques. In addition, some of them belonging to *Aspergillus* (*Aspergillus tamarii*) and *Penicillium* (*Penicillium carneum*, *Penicillium melanoconidium*, and *P. aurantiogriseum*) genera showed production of this mycotoxin by MECE and HPLC-MS despite the fact that they have never been described as OTA producers. This fact could be due to species misidentifications in the corresponding Culture Collections. However, after analysing the partial sequences of ITS region and *β-tubulin* gene reported by our research group for these strains (GenBank accession numbers JN097809, JN097810, JN097811, JN097812, JN097813, JN097814, JN217227, and JN217233), the identifications agreed with those reported by their respective Culture Collections in all of the above mentioned mould strains.

Regarding to the PCR using the designed primer pairs, the most appropriate was F1OT/R1OT based on the *otanps*PN gene since this primer pair generated a specific amplicon of 459 bp in all tested producing strains. These results were closely related to the detection of OTA production by MECE and HPLC-MS. In addition, the specificity of the primer pair F1OT/R1OT was confirmed since they match with the sequences from the *otanps*PN gene previously reported by our research group for several OTA-producing species that included all the different species and genera tested in this work (GenBank accession numbers JN097797, JN097798, JN097799, JN097800, JN097801, JN097802, JN097803, JN097804, JN097805, JN097806, JN097807, and JN097808). These results demonstrated the proper choice of the target sequence for designing primers capable of detecting different genera and species of OTA producers. From the point of view of food safety, this is an essential point in the design of a PCR method to detect toxigenic moulds. In spite of the fact that PCR protocols to detect specific OTA producing species have been reported (Bogs et al., 2006; Dao et al., 2005), they are not

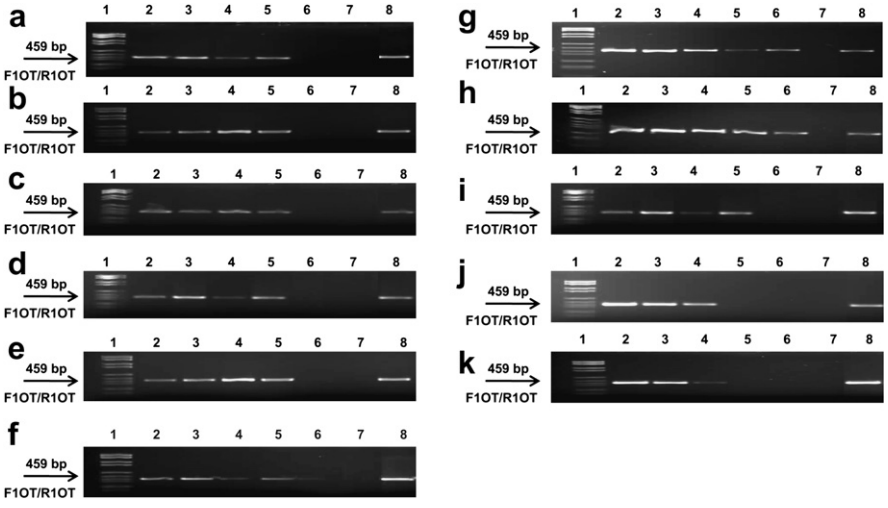

Fig. 3. Agarose gel electrophoresis of PCR products obtained using primer pair F1OT/R1OT in inhibition assays with food matrices: cooked turkey breast (a), cooked ham (b), mortadella (c), dry-cured ham (d), dry-fermented sausage "salchichón" (e), cured cheese (f), grape (g), plum (h), paprika (i), almond (j), and walnut (k). Lane 1: molecular weight marker of 2.1–0.15 kbp. Lanes 2–6: DNA template consisting of 5 μL of OTA producing moulds DNA from pure cultures mixed with 1, 2, 3, 4, and 5 μL of DNA from each non inoculated food product. Lane 7: 5 μL of DNA from non inoculated food samples. Lane 8: 5 μL of DNA from pure culture of OTA producing *Penicillium verrucosum* FHSC-3 (a–c), *Penicillium nordicum* CBS 110769 (d–f), and *Aspergillus ochraceus* CBS 589.68 (g–k).

Table 4
Detection of presence of fungal DNA and ochratoxigenic moulds in naturally infected dry-cured ham, paprika, and durum wheat semolina samples by PCR.

Food products						
Number of sample	Dry-cured ham		Paprika		Durum wheat semolina	
	PCR based on the *β-tubulin* gene	PCR based on the *otanps*PN gene	PCR based on the *β-tubulin* gene	PCR based on the *otanps*PN gene	PCR based on the *β-tubulin* gene	PCR based on the *otanps*PN gene
1	[a]+	[b]−	+	−	+	−
2	+	+	+	−	−	−
3	+	+	+	−	−	−
4	+	+	+	−	−	−
5	+	+	+	−	−	−
6	+	+	+	−	−	−
7	+	+	+	−	−	−
8	+	+	−	−	−	−
9	+	+	+	−	−	−
10	+	+	−	−	−	−
11	+	−	−	−	−	−
12	+	+	+	−	−	−
13	+	+	+	−	−	−
14	+	−	−	−	−	−
15	+	−	+	−	−	−

[a] Amplification was observed in PCR reactions based on *β-tubulin* or *otanps*PN genes.
[b] Amplification was not observed in PCR reactions based on *β-tubulin* or *otanps*PN genes.

able to detect ochratoxigenic moulds regardless of genus and species.

With regard to PCR sensitivity, the developed method was able to detect DNA from OTA producing strains down to 25 pg, which was considered as the detection limit. In addition, this method was also able to detect small amounts of target DNA (50 pg) in presence of high amount of non-targeted DNA (50 ng) which supposed a slightly increased of the detection limit. This fact could be justified by the interference of unspecific DNA. Thus, Mayer, Bagnara, Färber, and Geisen (2003) reported that when a low fungal DNA concentration is present, a high concentration of the unspecific DNA could act as an inhibitor, apparently by competition. The sensitivity results with pure DNA were similar to those reported in other PCR methods previously described to detect OTA producing moulds based on ITS regions (Patiño, Vázquez, González-Salgado, Gil, & González-Jaén, 2007). However for the aforementioned PCR protocols, it was necessary to optimize different PCR conditions in order to detect each OTA producing mould strain. Furthermore, none of the developed PCR methods allowed amplifying DNA from the main ochratoxigenic moulds. However, the PCR protocol designed in this study was able to detect different genera and species of ochratoxigenic moulds using the developed primer pair and PCR protocol.

When the ability of the PCR method for detecting OTA producing moulds on artificially contaminated food systems was evaluated, the detection levels were ranged between 10^2 and 10^4 cfu/g, depending on the tested food matrix. The lowest values of sensitivity of the PCR assay were found in almond and walnut. This fact could be due to the proteins from these nuts may interfere with enzymes which are necessary for the development of PCR, such as *Taq* polymerases, being consequently affected the sensitivity of amplification (Ausubel et al., 1998). Furthermore, the high content of lipids, polysaccharides, and polyphenols of almonds and walnuts has been reported as potential PCR inhibitors (Dickison, Kroll, & Grant, 1995). The lowest conidia levels detected in this study were similar to those found by Shapira, Paster, Menasherov, Mett, and Salomon (1996) and lower than those reported by Färber, Geisen, and Holzapfel (1997) both for aflatoxinogenic moulds using PCR protocols with previous incubation of samples.

Given that some inhibitory effects from tested food matrices in PCR reaction may be produced, an assay about the inhibition from the food components was carried out by parallel amplification of pure DNA of OTA producing moulds mixed with DNA extracted from non inoculated foods. A low influence of food components on the method sensitivity was observed since only the two highest levels of DNA (4 and 5 μL of DNA) from some of the non inoculated foods inhibited the PCR. Inhibition only with high amount of DNA from foods has also been reported in the detection of aflatoxigenic moulds in fresh figs (Färber et al., 1997).

The detection of ochratoxigenic moulds by PCR method in different naturally infected foods showed that up to 73% of dry-cured ham samples contained these moulds. This result is worrying since dry-cured ham is a ready-to-eat food and an inappropriate storage could trigger OTA production. In all dry-cured ham samples showing OTA producing moulds were detected also fungal contamination by *β-tubulin* PCR protocol. Thus, the use of this last protocol together with the designed PCR method to detect OTA producing moulds could be very important to avoid false PCR results. In paprika most of the tested samples showed fungal contamination according with the results of *β-tubulin* PCR protocol, although it was not due to the presence of OTA producing moulds. In wheat semolina, most of the samples tested did not show any fungal contamination and, none of them, showed OTA producing moulds. The low natural contamination by fungi found in the wheat semolina may be a consequence of the removal of the inner endosperm fraction during throughout the processing leading to less microbial contamination (Berghofer, Hocking, Miskelly, & Jansson, 2003). Although a higher number of natural contaminated samples should be tested, the above results together with those obtained in artificially contaminated food matrices allow validating the developed method to detect OTA producing strains in food samples.

The designed PCR method demonstrated to be reasonably rapid, as it enabled the detection of OTA producers in approximately 8 h (5 h for treatment and DNA extraction from foods, and 3 h for the analysis and display of PCR results). Other molecular methods, such as real time PCR (qPCR) (Selma, Martínez-Culebras, & Aznar, 2008), amplified fragments length polymorphism (AFLPs) (Schmidt, Ehrmann, Vogel, Taniwaki, & Niessen, 2003), and single strand conformational polymorphism (SSCP) (Rath & Ansorg, 2000), and even combination of molecular and biochemical techniques (Parenicová et al., 2001), described to detect these toxigenic moulds on food are usually more laborious than the PCR protocol developed in this study. In addition, they require specific equipment and

a sophisticated technical knowledge. Because of its simplicity, sensitivity, and specificity, the designed PCR protocol could be used by the food industry to routinely detect ochratoxigenic moulds and consequently to prevent hazardous levels of OTA in their products. The effective detection of low levels of OTA producing moulds would allow the classification of ingredients for either their urgent processing or for convenient storage. Thus, this routine control should be integrated for monitoring these toxigenic moulds in the HACCP systems of the industries.

5. Conclusion

The developed PCR method in this study proved to be a quick, specific, and sensitive tool for detecting ochratoxigenic moulds. Given that the detection limit found in this assay was quite low, this could be used for detecting ochratoxigenic moulds which contaminating foodstuffs. This technique may help to prevent the presence of OTA throughout the food chain since a control of the producing mould colonization could be performed at an early stage of the process. The PCR protocol could be even used routinely in the HACCP programs of the food industry.

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