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# Duplex real-time PCR method with internal amplification control for quantification of verrucosidin producing molds in dry-ripened foods

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ABSTRACT

Verrucosidin, which is a tremorgenic mycotoxin responsible for neurological diseases, has been detected in different dry-ripened foods as consequence of the growth of toxigenic molds. To improve food safety, the presence of verrucosidin producing molds in these kind foods should be quantified. The aim of this study was to design a duplex real-time PCR (qPCR) protocol based on TaqMan methodology with an internal amplification control (IAC). Eleven verrucosidin producing and 11 non producing strains belonging to different species often reported in food products were used. Verrucosidin production was tested by micellar electrokinetic capillary electrophoresis (MECE) and high-pressure liquid chromatography–mass spectrometry (HPLC–MS). A primer pair (VerF1/VerR1) and a TaqMan probe (*Verprobe*) were designed from the SVr1 probe sequence of a verrucosidin producing *Penicillium polonicum*. The conserved regions of the *β-tubulin* gene were used to design primers (TubF1/TubR1) and probe (*Tubprobe*) of the non-competitive IAC. The functionality of the developed method was demonstrated by the high linear relationship of the standard curves which relating Ct values and DNA template of the tested verrucosidin producers using the verrucosidin and IAC primers. The ability to quantify verrucosidin producers of the developed TaqMan assay in all artificially inoculated food samples was successful, with a minimum detection limit of 1 log cfu per gram of food. This qPCR protocol including an IAC could be very useful to quantify verrucosidin producing molds in dry-ripened foods avoiding false negative results. This method should be proposed to monitor the target molds in HACCP programs to prevent the risk of verrucosidin formation and consequently avoid its presence in the food chain.



## 1. Introduction

The environmental conditions found in meats and cheeses throughout their ripening process favor growth of a mold population composed mainly of *Penicillium* and *Aspergillus* species (Kure et al., 2004; Núñez et al., 1996). Several species belonging to the *Penicillium* genus, such as *Penicillium polonicum*, *Penicillium aurantiogriseum* and *Penicillium melanoconidium* isolated from the surface of dry-cured ham and other dry-ripened foods, may produce verrucosidin (Frisvad et al., 2004; Núñez et al., 1996, 2000, 2007; Sabater-Vilar et al., 2003; Sonjak et al., 2011), a tremorgenic mycotoxin responsible for neurological diseases (Knaus et al., 1994; Wilson et al., 1981). Verrucosidin has been detected in dry-fermented sausages (Sunesen and Stahnke, 2003) as consequence of the growth of these toxigenic mold species.

The early detection of verrucosidin producing species in dry-ripened food production, even before fungal development, is crucial to prevent contamination by verrucosidin during the drying and ripening process, since there is no current industrial process to detoxify foods contaminated with mycotoxins (Puel et al., 2007). For this purpose, nucleic acid methods, especially real-time quantitative PCR (qPCR), could be an appropriate alternative to traditional identification methods. These techniques are rapid, specific and highly sensitive enabling an accurate and sensitive quantification of target DNA (González-Salgado et al., 2009; Mulé et al., 2006) thus they could be applied to quantify verrucosidin producing molds.

On the other hand, to develop specific qPCR assays it is essential to use sensitive methods to measure the verrucosidin production from reference mold strains. Micellar electrokinetic capillary electrophoresis (MECE) and high-pressure liquid chromatography–mass spectrometry (HPLC–MS) have been reported as sensitive methods to detect verrucosidin production (Martín et al., 2004; Sosa et al., 2002).

The qPCR based on TaqMan technology that uses hydrolysis probe offers higher specificity and reliability of the diagnostic procedure than other technologies such as SYBR Green. Several qPCR assays have been reported for detection of toxigenic molds using DNA-associating dyes (SYBR Green) and fluorescently labeled oligoprobes (TaqMan) (Atoui et al., 2007; Fredlund et al., 2008; Nicolaisen et al., 2009; Rodríguez et al., 2011a, 2011b; Selma et al., 2008; Suanthie et al., 2009). However, no qPCR protocol has yet been developed to quantify verrucosidin producing molds in foods.

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Currently, no information is available about verrucosidin encoding genes in *P. polonicum* and other verrucosidin producing species to be used for designing specific primers and probe. An alternative may be the use of a DNA probe related to verrucosidin producing *P. polonicum* after a differential molecular screening procedure reported by Aranda et al. (2002). From this, DNA primers and probes for qPCR protocols may be designed.

Furthermore, the designed qPCR should be efficient in food samples. This efficiency may be reduced by the inhibitors from the food matrix (Di Pinto et al., 2007; Lucero Estrada et al., 2007; Mafra et al., 2008). Several organic compounds such as polysaccharides or fatty acids from ripened foods (Makhzami et al., 2008) may act as inhibitors of PCR and can also lead to errors in the specific detection of the verrucosidin producers (Monnet et al., 2006; Mulé et al., 2006). Therefore, direct testing in foods is required to evaluate the potential application of the qPCR based method for detection and quantification of molds. In addition, secondary metabolites produced by molds may be potential PCR inhibitors or generate mutant fungal strains lacking genes involved in mycotoxin production, leading in both cases to false negative in the PCR (Paterson, 2007; Paterson and Lima, 2009; Sant'Ana et al., 2010).

The use of an internal amplification control (IAC) is an adequate strategy to assess the validity of the PCR results and avoid false negative results derived from PCR inhibitors or from the generation of fungal mutations (Hoorfar et al., 2004; Paterson, 2007; Raymaekers et al., 2009). The guidelines for PCR testing of foodborne pathogens have proposed the presence of an IAC for PCR-based diagnostic tests (OECD; ISO, 22174:2005; ISO, 22119:2011). In such cases, a false negative qPCR result is a major hazard in the food industry. When an IAC is added to the qPCR, it is amplified together with the target sequence under the same conditions. Thus, it is important to optimize the concentration of the IAC to avoid the interference with the sensitivity of the assay (Lund and Mansen, 2006; Sachadyn and Kur, 1998). The use and development of IAC from housekeeping genes and a synthetic plasmid constructed for different microbiological purposes have been reported by several authors (Hartman et al., 2005; Khot et al., 2008; Randall et al., 2010; Rodríguez-Lázaro et al., 2005; Stöcher et al., 2002). The *β-tubulin* gene is suitable for designing primers and probes for non-competitive IAC since it is a housekeeping gene present in all eukaryotic cells (Cabañas et al., 2009; Campos et al., 2009; Rodrigues et al., 2009).

The aim of this work was to develop a sensitive and specific duplex qPCR method for detecting and quantifying verrucosidin producing molds in dry-ripened foods. The method included an IAC within the qPCR which co-amplified simultaneously in order to avoid false negative results. The efficiency and sensitivity of the developed method to quantify verrucosidin producing molds in foods were also analyzed.

## 2. Material and methods

### 2.1. Fungal strains

Twenty-two mold strains, belonging to 14 different species, were obtained from the Spanish Type Culture Collection (CECT), the Centraalbureau voor Schimmelcultures in The Netherlands (CBS), the Type Culture Collection of the Department of Biotechnology from the Technical University of Denmark (IBT) and the Culture Collection of Food Hygiene and Safety from the University of Extremadura. Only 8 of them were known as verrucosidin producers, whereas no information on verrucosidin production was available for the remaining strains (Table 1).

### 2.2. Verrucosidin production

Production of verrucosidin was tested after growing each mold strain, 3 inoculum points per plate, on Malt Extract Agar (MEA, 2% malt extract, 2% glucose, 0.1% peptone, and 2% agar) and incubating

**Table 1**
Verrucosidin production and qPCR data with the verrucosidin primers (VerF1 and VerR1) and *Verprobe* probe and the non-competitive IAC primers (TubF1/TubR1) and *Tubprobe* probe.

| Species designation | Strain reference | Information provided by culture collections | Verrucosidin production as detected by MECE/HPLC MS | qPCR amplification in TaqMan reactions $C_t \pm SD$[a] | IAC qPCR amplification $C_t \pm SD$ |
|---|---|---|---|---|---|
| *Aspergillus flavus* | CECT 2687[b] | Unknown | ND[c] | – | 20.7 ± 0.30 |
| *Aspergillus niger* | CECT 20157 | Unknown | ND | – | 20.3 ± 0.41 |
| *Aspergillus parasiticus* | CECT 2681 | Unknown | ND | – | 20.9 ± 0.08 |
| *Aspergillus versicolor* | CECT 2903 | Unknown | ND | – | 20.9 ± 0.13 |
| *Emericella nidulans* | CBS 465.65[d] | Unknown | +[e] | 22.3 ± 0.06 | 20.6 ± 0.03 |
| *Emericella quadrilineata* | CBS 235.65 | Unknown | + | 22.8 ± 0.52 | 20.4 ± 0.17 |
| *Penicillium aurantiogriseum* | CECT 2918 | Producer | + | 22.1 ± 0.32 | 20.1 ± 0.43 |
| *Penicillium aurantiogriseum* | CBS 112021 | Producer | + | 21.2 ± 0.03 | 20.0 ± 0.30 |
| *Penicillium aurantiogriseum* | CECT 2264 | Unknown | + | 22.0 ± 0.48 | 20.8 ± 0.32 |
| *Penicillium commune* | CBS 311.48 | Unknown | ND | – | 21.0 ± 0.05 |
| *Penicillium commune* | CBS 341.59 | Unknown | ND | – | 21.3 ± 0.17 |
| *Penicillium dipodomyicola* | CBS 110425 | Unknown | ND | – | 20.8 ± 0.02 |
| *Penicillium dipodomyicola* | CBS 110426 | Unknown | ND | – | 20.4 ± 0.06 |
| *Penicillium expansum* | CECT 2280 | Unknown | ND | – | 20.5 ± 0.28 |
| *Penicillium griseofulvum* | IBT 14319[f] | Unknown | ND | – | 20.6 ± 0.30 |
| *Penicillium melanoconidium* | CBS 64195 | Producer | + | 22.1 ± 0.18 | 20.9 ± 0.43 |
| *Penicillium melanoconidium* | CBS 109605 | Producer | + | 22.3 ± 0.32 | 21.1 ± 0.51 |
| *Penicillium polonicum* | Pc 10[g] | Producer | + | 22.4 ± 0.13 | 20.7 ± 0.23 |
| *Penicillium polonicum* | CBS 112490 | Producer | + | 20.0 ± 0.43 | 19.0 ± 0.30 |
| *Penicillium polonicum* | CBS 639.95 | Producer | + | 19.1 ± 0.07 | 18.2 ± 0.44 |
| *Penicillium polonicum* | CBS 101479 | Producer | + | 19.6 ± 0.18 | 18.9 ± 0.03 |
| *Penicillium viridicatum* | CECT 2320 | Unknown | ND | – | 20.3 ± 0.56 |

All verrucosidin producing strains are shaded.
[a] Data represent the mean threshold cycle (Ct) ± standard deviation (SD) of the 3 independent experiments each consisting of triplicate samples.
[b] CECT: Spanish Type Culture Collection.
[c] ND: verrucosidin production was not detected by MECE or HPLC–MS.
[d] CBS: Centraalbureau voor Schimmelcultures (The Netherlands).
[e] +: verrucosidin production was detected by MECE or HPLC–MS.
[f] IBT: Type Culture Collection of the Department of Biotechnology (Technical University of Denmark).
[g] Pc: strains isolated from dry-cured ham belonging to the Culture Collection of Food Hygiene and Safety from University of Extremadura.

for 15 days at 25 °C. The content of three petri dishes was extracted with chloroform, filtered twice through anhydrous sodium sulfate and evaporated in a rotatory evaporator at 40 °C as previously described by Sosa et al. (2002). The residue was resuspended in 5 mL of chloroform, filtered through a 0.45 μm pore size nylon membrane, and evaporated to dryness under a gentle stream of nitrogen. The extracts were stored at 4 °C in the dark until further use and resuspended in 200 μL of acetonitrile just before analysis. MECE analysis was carried out according to Martín et al. (2004) in a Beckman P/ACE 5500 model with a photodiode array detector (Beckman Instruments, Fullerton, USA). A fused silica capillary of 57 cm length and 75 μm 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 Sosa et al. (2002) in a Hewlett Packard series 1100 apparatus (Hewlett Packard, Palo Alto, USA). A Supelcosil LC-18 column (SUPELCO, Bellefonte, 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. Verrucosidin was identified in a Finnigan LCQ Mass Spectrometer (Finnigan, San Jose, USA) with atmospheric pressure chemical ionization source (APCI), according to its retention time and molecular mass.

### 2.3. DNA extraction

Every mold strain was 3-point inoculated on MEA and incubated for 4 days at 25 °C. The mycelium of each strain was scraped off the agar and used for genomic DNA extraction. For this, 50 mg of mycelium was digested with proteinase K and lyticase, frozen in liquid nitrogen and ground with mortar and pestle, prior to DNA extraction in a semiautomatic vacuum system following the method described by Sánchez et al. (2008). DNA concentration was quantified spectrophotometrically and 1.0 ng of DNA extracted from producing and non producing strains was used for qPCR assay.

### 2.4. Non-competitive internal amplification control

One IAC amplified only the partial *β-tubulin* gene with primers described by Glass and Donaldson (1995). An amplicon of approximately 453 bp was obtained from several reference strains belonging to different species of *Penicillium* and *Aspergillus*. These amplicons were purified and sequenced. A primer pair (TubF1/TubR1) and a TaqMan probe (*Tubprobe*) were then designed from conserved regions using Primer Express software (Applied Biosystems, Foster City, USA, Table 2). The probe was labeled at the 5′ end with the reporter indocarbocyanine (CY3) and at the 3′ end with the quencher Black Hole Quencher 2 (BHQ2).

**Table 2**
Nucleotide sequence of primers and probes used for duplex TaqMan qPCR assay.

| Primer/probe name | Nucleotide sequences (5′–3′) | Amplicon length | Position |
|---|---|---|---|
| VerF1 | CACGAAACGGAGACGC | 53 bp | 117[a] |
| VerR1 | AGAGACCACAGGAGCTTC | | 152 |
| TubF1 | TCCCTTCGGCAAGCTTTTC | 62 bp | 320[b] |
| TubR1 | TGTTACCAGCACCGGACTGA | | 363 |
| *Verprobe* | [HEX]-ATAAGTTTTGTGGAAAGCA-[BHQ1] | | 133 |
| *Tubprobe* | [CY3]-CGCCCCGACAACT-[BHQ2] | | 340 |

[a] Positions are in accordance with SVr1 probe sequence gene of *P. polonicum* CBS 222.90 (patent ES 2190755 B1).
[b] Positions are in accordance with published sequence of *β-tubulin* gene of *P. polonicum* (GenBank accession no. JN398145).

### 2.5. Primers and TaqMan probe to detect verrucosidin producing molds

A primer pair, (VerF1/VerR1) and a TaqMan probe (*Verprobe*) from the SVr1 probe sequence (patent ES, 2190755 B1, 2005) of a verrucosidin producing *P. polonicum* (Aranda et al., 2002) were designed by using the Primer Express software (Applied Biosystems, Table 2). The *Verprobe* was labeled at the 5′ end with the reporter hexachlorofluorescein (HEX) and at the 3′ end with the quencher Black Hole Quencher 1 (BHQ1).

### 2.6. Duplex TaqMan qPCR

The primers/probe set for the amplification of the verrucosidin producing molds was included together with primers/probe set of the IAC. Duplex qPCR was carried out in the Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems). qPCR was prepared in triplicate in a final volume of 25 μL reaction mixture in MicroAmp optical 96-well reaction plates and sealed with optical adhesive covers (Applied Biosystems). Three replicates of a control sample without DNA template were also included in all the runs. A mixture containing 5 μL of genomic DNA from the verrucosidin producing strain *P. polonicum* CBS 101479 was used to optimize the duplex qPCR method. For optimizing the concentration of primers and probes, several concentrations ranging from 50 to 600 nM were assayed. The optimized TaqMan protocol was carried out in a final volume of 25 μL, containing 5 μL of template DNA, 12.5 μL of 2x *Premix Ex Taq*™ (Takara, Otsu, Shiga, Japan), 0.5 μL of 50x ROX™ Reference Dye (Takara), 80 nM of both TubF1 and TubR1 primers and *Tubprobe*, and 400 nM of both VerF1 and VerR1 primers and *Verprobe*.

To optimize qPCR conditions, different annealing temperatures and times were assayed ranging from 57 to 62 °C and from 30 s to 2 min. The optimal thermal cycling conditions included an incubation of 2 min at 50 °C to allow the activation of uracil-*N*-glycosylase (UNG) enzyme, an incubation step for 10 min at 95 °C to denature the UNG enzyme and activate AmpliTaq Gold polymerase, 45 cycles at 95 °C for 15 s, 57 °C for 30 s and 60 °C for 30 s. Ct determinations were automatically performed by the instrument using default parameters. The size of the PCR products was verified by electrophoresis in 2.5% agarose gels.

### 2.7. Specificity of qPCR reaction

The specificity of the VerF1/VerR1 primers was tested using a fixed amount of 1.0 ng of genomic DNA from 22 strains of verrucosidin producing and non producing *Aspergillus*, *Emericella* and *Penicillium* species commonly found in foods (Table 1). The qPCR reactions were carried out as described in Section 2.6.

### 2.8. Sensitivity of qPCR reaction

The verrucosidin producing strain *P. polonicum* CBS 101479 was used to evaluate the sensitivity of the designed methods. For this purpose, 10-fold DNA dilutions of the above strain ranging from 10 ng to 0.001 pg were prepared. For qPCR amplification, 3 replicates of each DNA dilution were assayed per run. Standard deviations were calculated for each treatment between technical replicates. The detection limit of the producing strain was defined as the lowest DNA concentration detected in all reactions. The criteria considered for reliability of the designed methods was the correlation coefficient and the amplification efficiency calculated from the formula $E = 10^{-1/S} - 1$ (S being the slope of the linear fit).

### 2.9. Sensitivity of qPCR on artificially inoculated food matrices

The sensitivity of the optimized qPCR methods was assayed with DNA extracted from three kinds of non-sterile commercial vacuum

packed dry-ripened foods produced without protective molds (dry-cured ham, dry-fermented sausage "salchichón" and dry-ripened cheese). These products were inoculated with spores of different verrucosidin producing strains in order to evaluate the efficiency of the method. Thus, dry-cured ham was inoculated with *P. melanoconidium* CBS 109605, dry-fermented sausage "salchichón" with *P. aurantiogriseum* CECT 2918 and dry-ripened cheese with *P. polonicum* CBS 639.95. To test the specificity of both qPCR methods when applied on foods, the three types of foods were also inoculated with a non producing strain *P. dipodomyicola* CBS 110425.

The spores used for inoculation purposes were harvested by flooding 3 plates (20 days old) of MEA with 5 mL of sterile nanopure water containing 10% glycerol (Scharlau Chemie S.A., Barcelona, Spain), and rubbing the surface with a glass rod. The conidial suspension was filtered through Whatman paper No 1, diluted in sterile nanopure water, as necessary, and quantified by microscopy, using a Neubauer counting chamber. Aliquots of 1 mL containing from 2 to 6 log conidia/mL of each mold strain were immediately used to spike food samples in order to reach final levels of 0, 1, 2, 3 and 4 log cfu per gram of food. 5 g of spiked food samples were then treated for DNA extraction following the method described by Rodríguez et al. (2011b). All inoculations and extractions were performed by triplicate for each food. In addition, triplicates of non-inoculated negative control were included in each experiment.

For qPCR amplification, 3 replicates of 5 μL DNA extracted from the inoculated foods and non-inoculated negative controls were assayed per run. Standard curves were generated for each group of food products and the efficiencies for the standard curves were calculated.

To directly estimate the load of the inoculated mold, the samples were diluted in 0.1% peptone water, spread-plated on Potato Dextrose Agar (PDA, Scharlau Chemie S.A.) and incubated at 25 °C for 4 days. The natural fungal contamination of the samples was lower than 1 log cfu per gram. After characterizing the isolates from non-inoculated foods, no typical colonies of *P. melanoconidium*, *P. aurantiogriseum* and *P. polonicum* were found. In addition, DNA of these fungal isolates yielded negative results in the developed qPCR.

### 2.10. Quantification of fungal growth in artificially inoculated foods by qPCR

Slices of non-sterile commercial vacuum packed dry-cured ham, dry-fermented sausage "salchichón" and dry-ripened cheese, approximately 5 g, with a cut surface of 25 $cm^2$, were placed separately in presterilized orthogonal receptacles made of methacrylate, where the humidity was kept constant by a saturated KCl solution (water activity 0.97) placed at the bottom of the receptacles. The samples were inoculated on the surface with spores of the verrucosidin producing strain *P. polonicum* CBS 101479 at a concentration of 3 log cfu per gram. Sampling was carried out in triplicate from each at 0, 3, 5 and 10 days of incubation at 25 °C. Negative controls from non-inoculated dry-cured ham, dry-fermented sausage "salchichón" and dry-ripened cheese samples were also analyzed. DNA was extracted as described in Section 2.9 and subjected to the designed qPCR method as described in Section 2.6. The Ct values obtained with the TaqMan assay for the inoculated samples were used to estimate the fungal load from the corresponding standard curves.

To directly estimate the load of the inoculated mold, the samples were decimally diluted in 0.1% peptone water and spread-plated on PDA. Colonies showing the characteristic morphology of *P. polonicum* after incubation at 25 °C for 4 days were enumerated.

### 2.11. 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. Detection of verrucosidin by MECE and HPLC–MS

MECE and HPLC–MS analyses confirmed verrucosidin production in all the 8 strains identified as verrucosidin producers by the Culture Collections (Table 1). In addition, 3 strains with no information on verrucosidin production in the Culture Collections produced verrucosidin, whereas the 11 remaining strains did not produce detectable amounts of this mycotoxin.

### 3.2. Design of a non-competitive internal amplification control

The DNA fragment of 453 bp from *β-tubulin* gene of the different species was amplified and sequenced as indicated in Section 2.4 in order to build a non-competitive IAC. The obtained sequences were analyzed and deposited in GenBank matching with *β-tubulin* genes (GenBank accession no. JN097812, JN097813, JN217227, JN217228, JN217230, JN398140, JN398141, JN398142, JN398143, JN398144, JN398145). All these sequences were aligned using EMBL-EBI tools (http://www.ebi.ac.uk/). When the above sequences were compared with the published sequences of *β-tubulin* gene of different species, a similarity greater than 99% was always obtained.

### 3.3. Optimization of qPCR conditions

The best primers and probe concentrations showing the lowest Ct value with an adequate fluorescence for a given target concentration were selected for further analyses. Thus the lowest Ct value was obtained with 400 nM of each VerF1 and VerR1 primers and *Verprobe* probe and with 80 nM of each TubF1 and TubR1 primers and *Tubprobe* probe.

The optimization of the reaction conditions can reduce primer–dimer formation and increase the efficiency and specificity of the amplification process. The optimal thermal cycling conditions were 1 cycle of 2 min at 50 °C, 1 cycle of 10 min at 95 °C and 45 cycles at 95 °C for 15 s, 57 °C for 30 s and 60 °C for 30 s.

### 3.4. Specificity of the qPCR reactions using reference strains

The primers and probes specificity was tested on genomic DNA from the producing and non producing species belonging to *Aspergillus*, *Emericella* and *Penicillium* (Table 1). All verrucosidin producing reference mold strains detected by HPLC–MS and MECE analysis showed Ct values of $21.6 \pm 1.42$ with VerF1 and VerR1 primers and *Verprobe* probe, while no PCR amplification was observed in the non producing strains (Table 1). However, all producing and non producing reference mold strains showed Ct values of $20.4 \pm 0.80$ with TubF1 and TubR1 primers and *Tubprobe* probe (Table 1).

### 3.5. Standard curves, sensitivity and detection limits of duplex qPCR

The sensitivity of the qPCR assays was evaluated by testing 10-fold dilution series from 10 ng to 0.001 pg DNA from *P. polonicum* CBS 101479. The detection limit was 0.1 pg.

Standard curve relating the Ct values and the amount of *P. polonicum* CBS 101479 purified DNA was generated for the optimized method. A good linear relationship between the increasing Ct values and the target DNA was observed over the range from 10 ng to 0.1 pg. The slopes of the linear regression curves were −3.42 and −3.44 when verrucosidin and IAC primers were used, respectively. Therefore, the efficiencies of the qPCR assay were 96.1 and 95.3%, respectively. Furthermore, the correlation coefficients were always higher than 0.98.

### 3.6. Sensitivity of the qPCR assay on artificially inoculated food matrices

Standard curves using DNA extracted from inoculated foods were generated for each food matrix. The slopes of the linear regression equations in the qPCR assays with verrucosidin primers for dry-cured ham, dry-fermented sausage "salchichón" and dry-ripened cheese, were −3.16, −3.17 and −3.22, respectively, while with IAC primers the slope values for the former food products were −3.11, −3.29 and −3.27, respectively (Fig. 1). A good linear correlation ($R^2$) was also obtained over the range from 1 to 4 log cfu/g per reaction for all the food matrices (Fig. 1). The efficiencies values ranged from 104.4 to 107.2% for verrucosidin primers while for IAC primers these values ranged from 101.3 to 109.7%. The detection limit in all the inoculated foods was 1 log cfu/g.

No amplification (Ct = 45) with verrucosidin primers was obtained in the above food matrices inoculated with a non producing strain (*P. dipodomyicola* CBS 110425), while with IAC primers the slope values for dry-cured ham, dry-fermented sausage "salchichón" and dry-ripened cheese were −3.13, −3.32 and −3.28, respectively (data not shown).

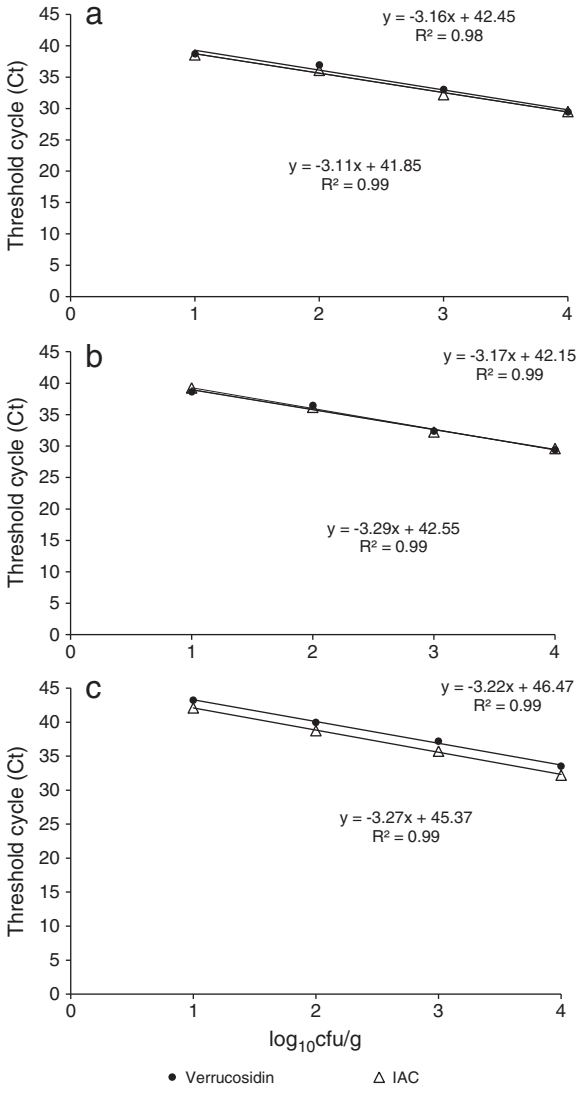


**Fig. 1.** Standard curves showing the log cfu/g vs. threshold cycle (Ct) values of duplex TaqMan qPCR method (with both VerF1/VerR1 and IAC primer pairs) for *P. melanoconidium* CBS 109605 inoculated in dry-cured ham (a), *P. aurantiogriseum* CECT 2918 inoculated in dry-fermented sausage "salchichón" (b) and *P. polonicum* CBS 639.95 inoculated in dry-ripened cheese (c).

### 3.7. Quantification of the fungal load in artificially inoculated foods

The ability of the designed qPCR method to quantify the fungal load in inoculated food samples after different incubation times is shown in Fig. 2. In addition, cfu/g values by plating were determined (Fig. 2). The growth of *P. polonicum* CBS 101479 differed slightly in the tested foods. In dry-ripened cheese, this strain started to grow after 1 day of incubation, whereas in dry-cured ham and dry-fermented sausage "salchichón" showed a 3 days lag phase. No natural fungal contamination was observed in non-inoculated food controls. Besides, when the DNA extracted from these control samples was tested by the duplex qPCR assay, no amplification was observed.

In general, the fungal load obtained by qPCR (with both VerF1/VerR1 and IAC primer pairs) correlated well with the cfu data obtaining always $R^2$ values around 0.98. Furthermore, the fungal load obtained by qPCR testing the verrucosidin detecting primers (VerF1/

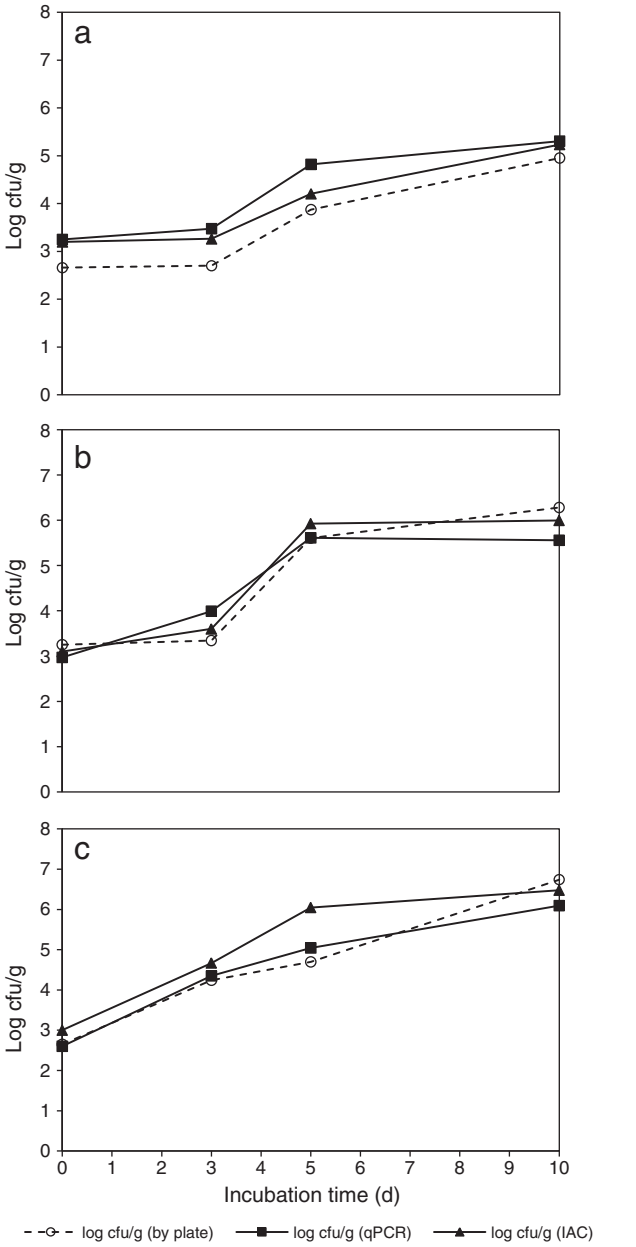


**Fig. 2.** Fungal count (log cfu/g) obtained by duplex qPCR (with both VerF1/VerR1 and IAC primer pairs) and by plate count of *P. polonicum* CBS 101479 inoculated in dry-cured ham (a), dry-fermented sausage "salchichón" (b) and dry-ripened cheese (c) during 10 days of incubation.

VerR1) was compared with counts obtained with the IAC from *β-tubulin* gene and high correlations of 0.97 ± 0.43, 0.97 ± 0.25 and 0.94 ± 0.70 for dry-ripened cheese, dry-fermented sausage "salchichón" and dry-cured ham were observed, respectively. In all the foods, duplex qPCR was able to quantify fungal load just after inoculation and before mycelium growth. No significant differences were found between fungal load determined by qPCR and by counting in PDA throughout the 10 days of incubation time. The standard deviation was lower than 0.20 in all phases of growth.

## 4. Discussion

In this study, a duplex qPCR assay including an IAC for detecting and quantifying verrucosidin producing molds has been developed.

The use of an IAC in diagnostic PCR is becoming mandatory (Hoorfar et al., 2003) because it could indicate errors caused by the presence of PCR inhibitors from the food matrix (Al-Soud and Rådström, 1998), culture (Paterson, 2007) or derived from the effect of secondary metabolites (Paterson and Lima, 2009; Sant'Ana et al., 2010). In addition, IAC could show errors due to thermocycler malfunction, incorrect PCR mixture or poor DNA polymerase activity (Hoorfar et al., 2003).

The development of an IAC based on conserved fungal sequences is an advantage because this offers reliable screening of cultured fungi. Due to the fact that such material (mycelium or spores) can also contain PCR inhibitors, its effect on PCR efficacy needs to be identified (Paterson, 2007). Thus, Kulik (2011) designed an IAC based on conserved fungal sequence of 5.8S rDNA which was used in TaqMan assay for reliable quantification of *Fusarium* chemotypes. In the present work, an IAC has been developed based on the highly conserved *β-tubulin* housekeeping gene. This target is essential for the structure and kinetics of the cytoskeleton (Campos et al., 2009) and it consists of a simple, easy and versatile solution for identifying false negative PCR results in the detection of toxigenic molds.

To develop specific qPCR assays for quantifying verrucosidin producing molds it is necessary to design specific primers and probes. To date, no verrucosidin related genes have been cloned. However, a DNA sequence constructed on the basis of DNA related to verrucosidin producing *P. polonicum* has been published (patent ES 2190755 B1, Aranda et al., 2002). The optimized method using the designed VerF1/VerR1 primer pair and *Verprobe* probe from the above DNA probe can be considered specific since it provided a good discrimination between verrucosidin producing and non producing strains.

The sensitivity of the developed method was also demonstrated by the high linear relationship of the standard curves constructed with 10-fold dilutions of DNA template and Ct values for a representative verrucosidin producing mold. Although no guidelines have been established for standard curves used in qPCR assays that quantify fungi, Fredlund et al. (2008) and Suanthie et al. (2009) suggested the use of criteria established for Genetically Modified Organism analysis of foods where the slope of the standard curve should range between −3.1 and −3.6, corresponding to a PCR efficiency of 80 and 110% respectively and the $R^2$ value be ≥0.98. These guidelines should also be valid for the analysis of fungal DNA in foods. In the present work, the optimized duplex method had $R^2$ values around 0.98. The slope values were in the acceptable range and similar efficiencies greater than 95% were obtained from verrucosidin and IAC primers, indicating a good optimization of the PCR reaction and that no errors occurred in this process. Similar values of efficiency have been reported for efficient qPCR protocols to quantify mycotoxin producing molds (Fredlund et al., 2008; Mulé et al., 2006; Rodríguez et al., 2011a, 2011b). Furthermore, the limit of detection, determined as the smallest amount of fungal DNA detected, was 0.1 pg, which is in the same range than other qPCR methods described in the literature for detecting toxigenic molds (Morello et al., 2007; Rodríguez et al., 2011b).

When the sensitivity of the qPCR assays was evaluated in different dry-ripened food matrices, all standard curves showed suitable linearity ($R^2 > 0.98$) and all slopes (with both primer pairs) were in the recommended range (Fredlund et al., 2008). The limit of detection in all inoculated foods, which was 1 log cfu per gram, was in the level of those previously reported (Rodríguez et al., 2011a, 2011b; Selma et al., 2008). Furthermore, no amplification with verrucosidin primers and amplification with IAC primers from non producing strain *P. dipodomyicola* CBS 110425 in inoculated foods demonstrated the robustness of the qPCR assays and the usefulness of including an IAC in qPCR to avoid false negative results.

The quantification of mold contamination in foods is a difficult task due to the nature of the fungal colony which consists of filamentous mycelia cells and single celled spores. Therefore, the assays to quantify the fungal load of target molds inoculated in dry-ripened cheese, dry-fermented sausage "salchichón" and dry-cured ham slices were designed to evaluate the efficiency of the developed method in complex matrices, where fungal biomass comprises hyphae as well as spores. The results of these assays throughout 10 days of incubation showed that the fungal load obtained by the qPCR method was nearly identical to the fungal load determined by plating. Similar results have been reported for other qPCR protocols developed to quantify the fungal load of patulin and aflatoxin producing molds in foods (Mayer et al., 2003; Rodríguez et al., 2011a).

The TaqMan qPCR procedure developed in the present study to quantify verrucosidin producing molds in dry-ripened foods could be performed within a relatively short time (5–6 h for DNA extraction and 1.5–2 h for qPCR). This is considerably lower than the time needed to quantify verrucosidin producing molds by conventional culturing techniques (3–5 days). This method thus enables reliable and rapid estimation of food contamination with verrucosidin producing molds. The fast duplex qPCR method designed including an IAC could be used to quantify the contamination of verrucosidin producing molds even before fungal development. Thus, this method could be very useful for evaluating the quality of raw materials/ingredients and monitoring the target molds in HACCP programs for preventing toxin production throughout the drying and ripening process of foods.

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