Food Control 21 (2010) 207–211

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Rapid detection and quantification of zearalenone-producing *Fusarium* species by targeting the zearalenone synthase gene *PKS4*

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ARTICLE INFO

Article history:
Received 22 October 2008
Received in revised form 15 May 2009
Accepted 25 May 2009

Keywords:
Fusarium
Zearalenone
Real-time PCR
SYBR Green I

ABSTRACT

This work is the first report on developing a method to detect and quantify the zearalenone-producing *Fusarium* species in foodstuff by real-time PCR assay with SYBR Green I. Based on the polyketide synthase gene (*PKS4*) sequences of four zearalenone-producing *Fusarium* strains, a specific primer set was designed and used to detect and quantify zearalenone-producing *Fusarium* in foodstuff. The system developed under study required only 100 mg infected maize flour for test, had ability to detect down to 10 copies of the target gene per reaction, and produced reliable quantitative data over three orders of magnitude. Compared with the conventional methods, it is more rapid, specific and sensitive to detect potential zearalenone contamination in food or feed production.

1. Introduction

Fusarium species are a group of soil, saprobic and phytopathogenic fungi. The fungus sometimes infects plants, causes *Fusarium* head blight, and results in severe grain yield losses. Some *Fusarium* species have been found to produce mycotoxins, such as fumonisin, trichothecene and zearalenone, and are considered as one of the most serious pathogenic filamentous fungi in contaminated foods or feedstuff. Zearalenone, a polyketide mycotoxin, has chronic estrogenic effects on mammals by causing reproductive problems in farm-raised pigs, experimental animals, livestock and human (Gaffoor & Trail, 2006; Toppari, et al.,1996). Currently, such methods like high performance liquid chromatography (HPLC), gas chromatography (GC) or liquid chromatography with tandem mass spectrometry (LC–MS/MS) are generally used to detect zearalenone (Labuda, Parich, Berthiller, & Tancinová, 2005; Lea & Christoph, 2003; Songsermsakul, Sontag, Cichna-Markl, Zentek, & Razzazi, 2006). Obviously, these methods are time-consuming and laborious. Therefore, a rapid and sensitive method to detect zearalenone-producing *Fusarium* species in stored grain will be valuable to predict the potential zearalenone contamination and thus be useful for further food quality monitoring as well as disease prevention and control.

Traditional methods to identify *Fusarium* species mainly depends on the morphologic characters of degenerate mycelia or symptoms on infected plant hosts. Though these methods are relatively simple, they are inefficient and time-consuming since the accuracy of the results depends on good experience to a large extent. Recently, numerous PCR-based methods for diagnosis and quantification of *Fusarium* species producing B-trichothecenes deoxynivalenol, fumonisin and nivalenol have been intensively described (Niessen, 2007), whereas detection and quantification of zearalenone-producing *Fusarium* strains has not been reported so far. In this regard, the present work aimed at developing a rapid and specific method for detection of zearalenone-producing *Fusarium* species in potential contaminated foodstuff. The real-time PCR system with SYBR Green I was adopted, and a fragment derived from the polyketide synthase gene *PKS4*, which is involved in zearalenone biosynthesis, was used as the specific target sequence. Meanwhile, maize flour was taken as a very commonly used grain material for detection.

2. Materials and methods

2.1. Fungal strains and culture conditions

Four zearalenone producers, *Fusarium culmorum* (DSM 1094), *Fusarium graminearum* (DSM 1095), *F. graminearum* (DSM 1096) and *Fusarium crookwellense* (JCM 9874), were purchased from the culture collection of the German Collection of Microorganisms and Cell Cultures (DSMZ) and Japan Collection of Microorganisms (JCM), respectively. Five non-zearalenone-producing *Fusarium* strains, *Fusarium argillaceum* (ACCC 30063), *Fusarium nioace* (ACCC

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doi:10.1016/j.foodcont.2009.05.014

30074), *Fusarium ortuoaras* (ACCC 30075), *Fusarium reticulatum* (ACCC 30077), *Fusarium sambucinum* (ACCC 30078), and 15 food relevant fungal strains, *Aspergillus flavus* (ACCC 31719), *A. flavus* (ACCC 30899), *A. flavus* (ACCC 30321), *Aspergillus fumigatu* (ACCC 30367), *A. fumigatu* (ACCC 30797), *Aspergillus nidulans* (ACCC 30469), *Aspergillus ochraceus* (ACCC 31749), *A. ochraceus* (ACCC 30471), *Aspergillus versicolor* (ACCC 31948), *A. versicolor* (ACCC 32160), *Beauveria bassiana* (ACCC 30808), *Paecilomyces islandicum* (ACCC 31950), *Penicillium citrinum Thom* (ACCC 31565), *Penicillium cyclopium* (ACCC 30485) and *Penicillium expansum* (ACCC 30904), were kindly provided by Agricultural Culture Collection of China (ACCC) as the controls. All strains were grown in potato dextrose agar medium (PDA) plates at 20 °C for maintenance or in PDA liquid culture under shaking conditions (200 rpm) at the same temperature for DNA extraction.

2.2. DNA extraction from pure culture or contaminated maize flour

DNA from pure culture or contaminated maize flour were all isolated using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) combined with a bead milling method as described previously (Dean, Betancourt, & Menetrez, 2004). One hundred milligram of mycelia or maize flour was suspended in 0.4 ml AP1 buffer of the Kit in a 2 ml screw cap conical tube preloaded with 0.3 g autoclaved glass beads (300 μm). The suspension was then mixed on a Mini Bead Beater™ (Biospec Products, Bartlesville, OK, USA) for 45 s at the maximal rate. The milling procedure was repeated for two more times after cooling the tube on ice for 1 min. The following spin column procedure was performed essentially according to the manufacturer's recommendations. In the last elution step, the DNA samples were eluted with 100 μl of ddH_2O and precipitated at −20 °C for 2 h after addition of two volumes of ice-cold 100% ethanol and 0.1 volume of 3 M sodium acetate. The precipitate was collected by centrifugation at 12,000 rpm for 20 min, dissolved in 20 μl of ddH_2O, and then stored at −20 °C before use. Two milliliter of each stock solution was used as the template in the quantitative real-time PCR reaction.

2.3. Contamination studies

Four target strains of *F. crookwellense*, *F. culmorum* and *F. graminearum* were inoculated on PDA slants for 4–8 weeks, and spores were harvested as previously reported (Dean, Roop, Betancourt, & Menetrez, 2005). The number of spores were determined by hemocytometer counting (Sambrook, Fritsch, & Maniatis, 1989) and further identified by plating standard 10-fold serial dilutions on PDA medium. Maize flour stored in our laboratory was inoculated with freshly prepared spores of these four *Fusarium* strains at the concentrations of 10^4, 10^3 and 10^2 spores per 100 mg of maize flour, respectively. Total DNA of each contaminated sample was extracted immediately as described above.

2.4. Primer design and target fragment isolation

Based on the gene sequence of polyketide synthase *PKS4* (GenBank accession number DQ019316), the forward primer F1 (5′-cgtcttcgagaagatgacat-3′) and the reverse primer R1 (5′-tgttctgcaagcactccga-3′) were designed by Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). Sequence specificity of the primers was checked by blastn program (http://www.ncbi.nlm.nih.gov/BLAST/). For isolation of the target gene fragment of *PKS4*, a conventional PCR reaction with the genomic DNA of four zearalenone-producing *Fusarium* strains as template was carried out using a thermocycler (Eppendorf, Hamburg, Germany). The reaction system contained template DNA 2 μl (10 μg/ml), 10× PCR buffer 2 μl, dNTP mix 4 μl (2.5 mM each nucleotide), primer F1 and R1 1 μl each (10 μM), Taq polymerase 1 U (TaKaRa, Otsu-shi, Shiga, Japan), and ddH_2O up to 20 μl. The PCR conditions were: 95 °C for 3 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 7 min. The PCR products about 0.28 kb fragment designated *PKS4F* were recovered using an Agarose Gel DNA Purification Kit (TaKaRa). The purified fragment was ligated into a T-easy 3 vector (TransaGen Biotech, Beijing, China), and then transformed into *Escherichia coli* JM 109. The DNA of plasmid harboring positive clones was purified using MiniBest Plasmid Purification Kit (TaKaRa) for subsequent sequencing. The plasmids containing the target fragment was then used as standard. The DNA concentration was determined using a Specord 205 spectrophotometer (Analytik Jena, Coston, UK) and the plasmid copy number was calculated. A series of 10-fold dilutions of stock solution were prepared as the templates for generating a standard curve through real-time PCR reactions.

2.5. Analysis of the specificity of the primer set

To test specificity of the F1/R1 primer set for the target fungi, RCR reactions were performed with the genomic DNA of five non-zearalenone-producing *Fusarium* strains, and 15 food relevant fungi. The composition of the PCR system and PCR conditions were the same as described above.

2.6. Real-time PCR

Real-time PCR reactions were performed in an iCycler iQ4 Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The PCR thermal cycling conditions were as follows: 95 °C for 6 m, 45 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and 80 °C for 10 s (during which the fluorescence was measured), and final extension at 72 °C for 7 min. Following the final amplification cycle, a melting curve was constructed by measuring the fluorescence continuously when heating from 65 to 95 °C at the rate of 0.5 °C per s. The PCR reaction contained 10 μl of SYBR Green PCR master mix (2×, Toyobo, Osaka, Japan), 1 μl of each primer (10 μM), 2 μl of template DNA, and 6 μl of ddH_2O up to a final volume of 20 μl. To generate the standard curve, 10-fold serial dilutions representing 10^7–10^1 copies of the target fragment *PKS4F* were prepared, and aliquots of each dilution were used as templates. A reaction with only ddH_2O as template was set as negative control. To quantify the copy number of *PKS4F* in contaminated feed samples, 2 μl of DNA stock solution of each sample was used as template. Amplification was performed under the same conditions described above and each sample was amplified in triplicate. Quantification values were automatically determined by the optical system software version 3.1 (Bio-Rad). The Ct values obtained were exported into a Microsoft Excel Worksheet for statistical analysis and then the gene copy number of each sample was calculated according to the generated standard curve.

3. Results and discussion

3.1. Specificity identification

Genes involved in biosynthesis and regulation of mycotoxins were preferred to be selected as the sources for primer design (Niessen, 2007). The *PKS4* gene of *F. graminearum* has been reported to be essential in the production of zearalenone (Lysøe, et al., 2006). By generation of *PKS4* single insertional replacement mutant, it was identified that the gene catalyzes critical steps in the synthesis zearalenone, and its product stimulates expression of another gene involved in the zearalenone synthetic pathway. Therefore, *PKS4* was selected as the target gene and a primer set

Table 1
Specificity demonstration of the primer set F1/R1with four various *Fusarium* species and food relevant fungal species.

Fusarium species	PCR results	Food relevant fungal species	PCR results
F. crookwellense	+	*A. flavus* (3)[c]	–
F. culmorum	+	*A. fumigatu* (2)[c]	–
F. graminearum[a]	+	*A. nidulans*	–
F. graminearum[b]	+	*A. ochraceus* (2)[c]	–
F. argillaceum	–	*A. versicolor* (2)[c]	–
F. nioace	–	*B. bassiana*	–
F. ortuoaras	–	*P. islandicum*	–
F. reticulatum	–	*P. citrinum Thom*	–
F. sambucinum	–	*P. cyclopium*	–
		P. expansum	–

[a] *F. graminearum* (DSM 1095).
[b] *F. graminearum* (DSM 1096).
[c] The number of different strains of the same fungal species are shown in parentheses.

F1/R1 was designed in this study. Because SYBR Green I dye is able to bind all of the PCR products including the nonspecific products, thus the specificity of the amplicons should be tested. Four fragments about 0.28 kb were amplified using the genomic DNA of four zearalenone-producing *Fusarium* strains as templates (Table 1). The results of sequencing and NCBI BLAST indicated that four fragments were all 279 bp in length and shared significant sequence identity (more than 99%) with the target region of *PKS4* (6198–6476 bp), and no significant similarity was found to any sequence from other organisms. Thus the plasmid harboring the 279 bp fragment of *PKS4* gene was used as the specific DNA standard to generate the standard curve for copy number determination. No amplification products were observed by templates from the 20 control strains (Table 1). This result proved that the target sequence was specific for the zearalenone-producing *F. crookwellense* and *F. culmorum* as well as the previously reported *F. graminearum*. Further BLAST analysis revealed that no transcript sequence completely matched the sequences of the primers simultaneously, suggesting primers F1 and R1 were unique.

3.2. Standard curve and sensitivity

A serial dilution of purified DNA templates was used for calibration of the system. The standard curve was generated based on the linear relationship between the input copy numbers of the template and the Ct values for the *PKS4* gene fragment amplifications over seven logarithmic dilutions (Fig. 1). There is a good correlation between template concentrations and Ct values (Fig. 2). Sensitivity of the system allowed detection down to 10 copies of DNA template. Specificity and efficiency are two essential requirements for successful real-time PCR amplification. Interference of nonspecific products and primer-dimers can dramatically affect the quality of the data. In the real-time PCR program, the temperature at which the fluorescence was read during each cycle was adjusted to 80 °C to prevent false positive measurements. PCR amplifications with different amounts of template DNA were analyzed by melting curve. Only one melting peak was observed at 89 °C, indicating that the total fluorescent signal was contributed by the majority of specific amplicons (Fig. 3). The negative control gave no fluorescent signal in the reactions. This result demonstrated that the primer set F1/R1 was functional to the target *PKS4* gene and specific for producing a single predominant product with a distinct Tm.

3.3. Maize sample analysis

The data of the biomass quantification of four zearalenone-producing strains in the artificially contaminated maize samples were shown in Table 2. No signal was measured in the uninoculated maize sample which used as the negative control. The CFU values determined by plate counting and the numbers of target gene copy quantified by real-time PCR were of the same order of magnitude over three 10-fold serial dilutions. However, the ratio between them was not exact one-to-one. The gene copy numbers determined were always higher than the CFU values observed. This difference might be ascribed to the special spore morphogenesis of *Fusarium* species. The macroconidia of the four zearalenone-producing *Fusarium* strains under study are morphologically similar, containing 3–6 septate cells (Burgess, Nelson, & Toussoun, 1982; Sugiura, Saito, Tanaka, Ichinoe, & Ueno, 1994), and one macroconidia contains at least two single nuclei for spore germination (Steven, 2005). Therefore, the target gene in one macroconidia would be two or more copies. If the genomic DNA was recovered completely, the target gene numbers quantified in one sample would be at least two times of its CFU number. In this study, the

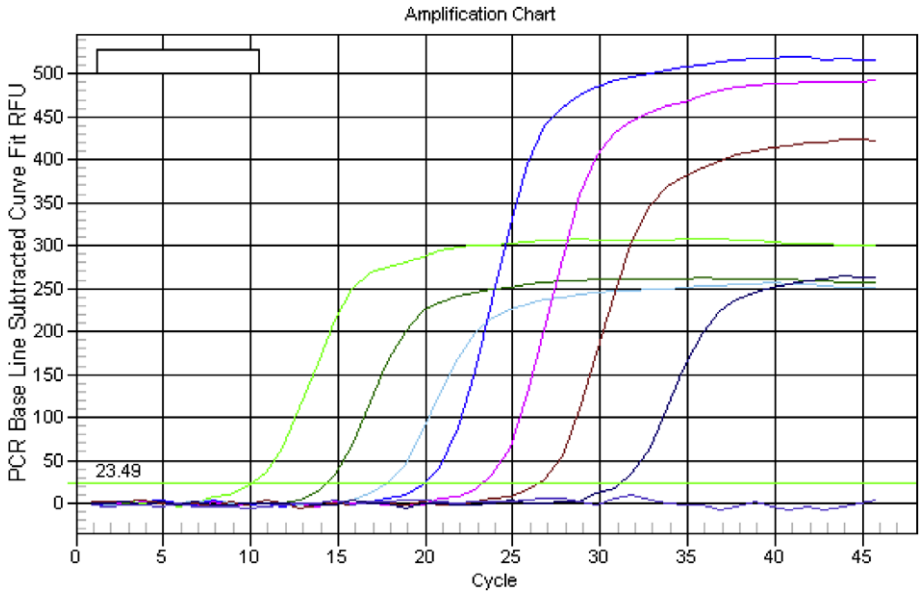

Fig. 1. Fluorescence kinetics of PCR with different standard plasmid DNA concentrations (10^7–10^1 copies).

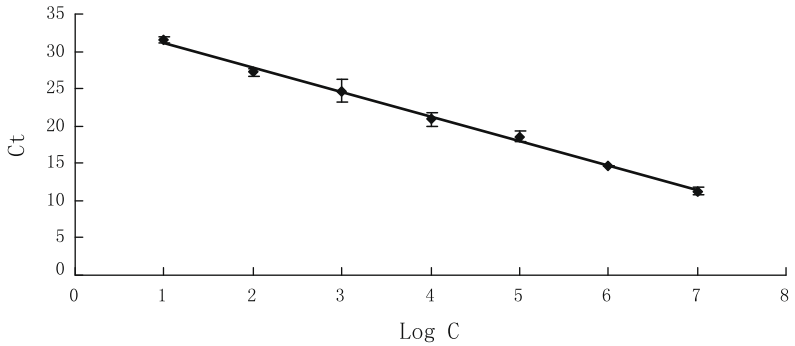

Fig. 2. Standard curve generated with two independent reactions showing the correlation between cycle number (Ct) and logarithmic concentration of *PKS4F* standard DNA (Log C). Slope: −3.3, intercept: 34.5, correlation: 0.996.

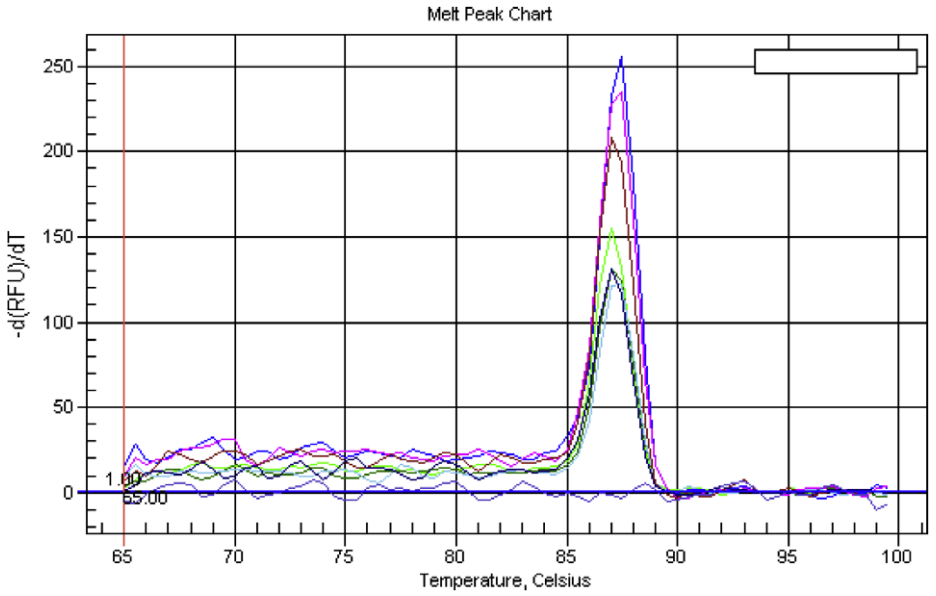

Fig. 3. Melting curves of specific amplificons from plasmid containing the target fragment *PKS4F* at different concentrations.

contaminated samples were prepared by inoculation of 10-fold diluted spores, thus the difference between the corresponding logarithm values of *PKS4* copies and CFU numbers in each sample would be 0.3 or more than 0.3. Among the twelve samples, seven samples showed a logarithm difference of greater than 0.3, this result truly reflected the typical microbial structure model of multinucleate macroconidia from *Fusarium* species. In the other five samples, although part of the DNA was not recovered completely, multiple nuclei in macroconidia cells still contributed to the increase of *PKS4* copy number at a higher rate than the CFU number, thus the number of the target gene quantified was still higher than the observed number of spores. Similar phenomenon has been reported by Mayer, Bagnara, Farber, and Geisen (2003), but the reason was mycelium which also contains multiple nuclei. In their real-time PCR assay to quantify *A. flavus* in infected foods, the existence of multinucleate mycelial cells in samples resulted in the difference of about 1–4 log units between the real-time PCR data and CFU numbers. Compared with their results, the maximum difference determined in this study was only 0.38 log units, suggesting that no mycelia contamination occurred and the CFU numbers in each sample could be effectively estimated by the corresponding real-time PCR values. Another advantage of this method is the manipulation of small-amount samples assay. Only 100 mg of a sample is required for the target organism analysis, which avoids laborious work for treatment of large amount of samples.

This quantitative PCR assay can effectively detect 10 copies of the target sequence per reaction. Using this system, about 500 CFU of the target fungus can be detected in 1 g of the sample, which is significantly lower than the minimum limit of phytopathogenic fungi detection (1000 CFU/g) for Hygienical standard for feeds in China national standard GB 13078–2001 (http://www.standardcn.com/standard_plan/list_standard_content.asp? stand_id=GB@13078-2001). Detection or screening of zearalenone-producing *Fusarium* species have been reported (El-Kady & El-Maraghy, 1982; Hacking, Rosser, & Dervish,

Table 2
The number of *Fusarium* spp. spores inoculated in the maize samples and *PKS4* gene copy numbers determined by real-time PCR.

Tested fungus	No. of CFU			No. of gene copy		
	10^2	10^3	10^4	10^2	10^3	10^4
F. crookwellense	2.18	3.18	4.18	2.27	3.25	4.56
F. culmorum	2.11	3.11	4.11	2.45	3.45	4.42
F. graminearum DSM 1095	2.06	3.06	4.06	2.39	3.24	4.28
F. graminearum DSM 1096	1.98	2.98	3.98	2.07	3.36	4.36

Ten-fold serial dilutions of spores were prepared for inoculation by hemocytometer counting. The logarithm values of CFU number was identified by plate counting and the Log *PKS4* gene copy was determined by real-time PCR. Samples included triplicate analysis.

1976). However, the methods used were all based on zearalenone analysis which was time-consuming and laborious. Generally, if zearalenone can be detected from food or feed stuff under practical conditions, the contamination has been very severe and the contaminated materials have to be discarded. Therefore it is of great importance to monitor the zearalenone-producing *Fusarium* species in advance to avoid potential zearalenone contamination in foodstuff.

4. Conclusions

In this study, a detection system based on quantitative real-time PCR was developed. It was proved to be rapid, sensitive, specific, cheap and reliable for identification and quantification of zearalenone-producing fungi in maize flour. This work is a prospective study on microbiological risk assessment of grain products, further optimization of this system is still required for its practical application in food or feed stuff safety evaluation.

Acknowledgements

This work was supported by the Chinese Program on Research for Public Good (No. 2005DIB4J038) and 948 Program of the Ministry of Agriculture (Grant No. 2007-Z3).

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