

Detection of trichothecene producing *Fusarium* spp. by PCR: adaptation, validation and application to fast food

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Abstract

Background. Food contamination by trichothecene mycotoxins is considered to be an emerging public health problem. The aim of this study was to validate a rapid sonification protocol, previously set up for cereal *Fusarium* DNA extraction from fast food samples, produced by a centre for research and development in the food industry in Catania, Sicily, and to validate it for a diagnostic PCR assay targeted at *tri5*, the key gene of trichothecene biosynthesis.

Methods. DNA from reference *Fusarium* spp. strains and from fast food samples was prepared, setting up an extraction protocol adapted using some modifications based on a method recently described. Validation experiments were performed: serial dilution of DNA extracted from fungal cultures were added to food samples and then DNA was extracted. Specific primer pairs were used to detect *F. graminearum* and *F. culmorum* DNA in species-specific assays as well as trichothecene-producing *Fusarium* spp. in a group-specific system.

Results. All genomic DNA extracted from trichothecene-producing *Fusarium* spp. as well as from DNA-spiked fast food samples and from food still in its original condition resulted in the correct amplification. The detection limit was 1×10^{-4} µg of DNA. All fungal and food samples tested gave highly consistent results in repeatability assays, thus demonstrating the within-lab and within/between-day precision of the method.

Conclusions. Information on the epidemiology of trichothecene producing *Fusarium* through the food chain and the identification of the most frequently contaminated components of fast food are essential in order to develop effective public health strategies for minimising consumer exposure to trichothecenes.

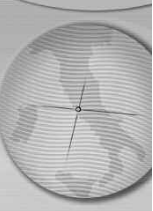
Key words: *Fusarium*, fast food, trichothecenes, PCR

Introduction

Fusarium species are important toxigenic fungi from temperate regions [1] and are found worldwide in cereals and other food types for human and animal consumption. Species such as *F. graminearum*, *F. culmorum*, *F. poae*, and *F. sporotrichioides* produce mycotoxins, such as trichothecenes deoxynivalenol (DON), nivalenol (NIV), diacetoxyscripenol (DAS) and T-2 toxin, as well as polyketide estrogen analogue zearalenone (ZEA), all of which are predominant in cereals worldwide.[2] Ingestion of infected grains or cereal products containing *Fusarium* mycotoxins may cause acute or chronic effects in humans and animals.[3] The first recognized trichothecene mycotoxicosis was an outbreak of alimentary toxic aleukia in the USSR in 1932, a hemorrhagic syndrome characterized by nausea, vomiting, diarrhea, dermatitis, and extensive internal bleeding; the mortality rate was 60%.[4] There are

some reports that indicate that trichothecenes may have been used as an agent for chemical warfare in South-East Asia (Lao People's Democratic Republic and Cambodia).[5,6] Common manifestations of trichothecene toxicity are depression of the immune responses, nausea and sometimes vomiting. A recent large-scale European study on the occurrence of *Fusarium* toxins (trichothecenes, fumonisins and zearalenone) [7] demonstrated that 57% and 20% of samples, mostly tested from North European countries, were positive for DON and T-2, respectively. A high frequency of DON was found in maize (89%) and wheat (61%). Thus, food contamination by trichothecene mycotoxins is considered an emerging public health problem.

Many mycotoxins are stable under normal food processing conditions and can therefore be present not only in food and animal feed but also in processed products: surveillance of retail food



and drinks, which are cereal in origin, show that trichothecenes are frequently present in the food and drinks consumed in Europe and survive the current food manufacture processes.[8] The food industry, nowadays often addressing the consumers and catering companies needs for easy and quick preparation products, the so-called fast food, requires measures to control trichothecene contamination and improved methods to ascertain their high quality standards. The analytical difficulty and the economic and public health importance of controlling trichothecenes in food supports the need for certified reference materials and validated methodologies.[9]

One such methodology is the application of rapid assays based on polymerase chain reaction (PCR) which can analyse samples for potential contamination with *Fusarium* species producing trichothecenes.[10] Species-specific PCR detection of *E. graminearum* [11] and group-specific PCR detection of trichothecene-producing *Fusarium* spp. [12] has been successfully demonstrated. Furthermore, a protocol was set up for the rapid preparation of fungal DNA from cereals.[13]

The aim of the present study was to validate the rapid sonification protocol set up by Knoll et al. [13] for *Fusarium* DNA extraction from fast food samples produced by a centre for research and development in the food industry in Catania, Sicily, and to validate it for a diagnostic PCR assay targeted at *tri5*, the key gene of trichothecene biosynthesis.

Methods

Organisms, media, culture condition

All reference *Fusarium* spp. strains (the kind gift from Dr. L. Niessen) used in this study, producing or not trichothecenes, are listed in Table 1. All fungal specimens were inoculated by stock cultures and grown on SNA medium [14] as working cultures. Fungal liquid cultures were grown in 3% malt extract broth containing 0.3% soy peptone in 15 ml vials at room temperature for 4-7 days.

Table 1. Organisms used in this study and the presence of a 658 bp PCR product with primers Tox5-1/Tox5-2.

Fungal species	Strain	PCR-reaction
<i>F. tricinctum</i>	DSM 62446	-
<i>F. avenaceum</i>	TMW 4.0281	-
<i>F. poae</i>	DSM 62376	+
<i>F. graminearum</i>	DSM 62376	+
<i>F. culmorum</i>	DSM 62376	+

Food samples

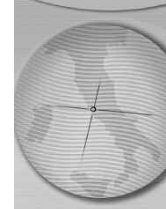
Food samples were from a centre for research and development in the food industry in Catania, Sicily, whose activity focuses on biological and natural products, to be freeze-dried, de-hydrated and desiccated, without any preserving/colouring or additives. Two food products were chosen to be contaminated by *Fusarium* spp. as their recipes include cereal components: "Prontocone", (wheat-flour, sugar, hydrogenated vegetal fat, maize-starch, eggs, skimmed milk, salt, soya lecithin, natural flavours) and "Prontocrepes" (wheat-flour, eggs, skimmed milk, hydrogenated vegetal fat, soya flour, sugar, salt, soya lecithin, vanilline), both were freeze-dried and dehydrated.

Preparation of DNA from pure fungal cultures

Our protocol was modified from the one described by Knoll et al.[13] All reference *Fusarium* spp. were cultured at room temperature in 3 ml of liquid medium in 15 ml vials until matured (4-7 days). Then, the mycelium was centrifuged and washed twice with sterile tap water; 3.3 ml of lysis buffer S3 and 6.7 ml distilled water were added and mycelia were sonicated for 1 minute with a small sonication tip using a model 150 Artek Sonic Dismembrator (energy density = 80 W cm²); buffer S3 consisted of 66 mM EDTA, 33 mM Tris, 3,3% Triton X-100, 1.65 M guanidinium-HCl, 0.825 M NaCl, 6% polyvinylpyrrolidone-40T, ddH₂O, adjusted to a pH of 7.9. Following sonification, 0.8 ml were taken from the supernatant and 0.4 vol of absolute ethanol was added. This mixture was spun through DNA extraction columns supplied with the High Pure PCR Template Preparation Kit (Roche, Mannheim) according to the manufacturer's recommendations. DNA was eluted with two rinses of 100 µl elution buffer, preheated to 70 °C, into a sterile 1.5 ml Eppendorf tube.

Preparation of DNA from "Prontocone" and "Prontocrepes"

DNA was extracted from 0.2 g of the fast food samples "Prontocone" and "Prontocrepes" using a commercially available kit, NucleoSpin Food kit (Macherey-Nagel GmbH & Co. KG, Germany). The extraction protocol suggested by the producer was adapted using some modifications based on a protocol recently described.[13] Briefly, 0.2 g of the fast food samples were mixed with 550 µl of lysis-buffer CF. Samples were sonicated for 1 minute using a model 150 Artek Sonic Dismembrator (energy density = 80 W cm²) equipped with a 3 mm diameter sonotrode. Following sonification, 10 µl of proteinase K was



added and the mixture was incubated at 65°C for 30 minutes. After centrifugation, 300 µl of clear supernatant were pipetted into a new 1.5 ml Eppendorf tube; after adding 300 µl of guanidium hydrochloride buffer and 200 µl ethanol the mixture was spun through the Nucleospin Food extraction column and the extraction was performed according to the manufacturer's recommendation.

In order to validate our DNA extraction method, serial dilution of 1 µg of DNA from fungal pure cultures of *F. poae* were added to 0.2 g of food samples and DNA was extracted using the protocol described above.

PCR protocol for the detection of genomic DNA of trichothecene-producing *Fusarium* species in pure fungal cultures and in fast food samples

Specific primer pairs were used, in single reactions, to detect *F. graminearum* and *F. culmorum* DNA in species-specific assays as well as trichothecene-producing *Fusarium* spp. in a group-specific system, as previously described although in a multiplex format.[15] The primer pair Tox5-1 and Tox5-2 was specific for all trichothecene producing *Fusarium* species, the primer pair GaoA V2 and GaoA R2 was species-specific for *F. graminearum* and the Opt18 R and Opt18 V was species-specific for *F. culmorum*. [13,16] Each PCR reaction was performed in a 25 µl volume using the puRe Taq Ready-to-go PCR beads (Amersham Biosciences), with 1 µl DNA template per reaction, 25 pmol of each of the following primers: GaoA V2 and GaoA R2, Opt18 R and Opt18 V, Tox5-1 and Tox5-2 which was then made up to final volume with molecular biology grade water. Water was used instead of DNA as a negative control for PCR in each experiment unless otherwise stated. PCR reactions were run on a Mastercycler Gradient (Eppendorf) under the previously described conditions.[13] All electrophoresis was performed using 1.5% horizontal agarose gels prepared with 0.5X TBE buffer and run in the same buffer at 120V. Gels were stained with ethidium bromide and documented by the GeneScan Analysis Software (PE Biosystem).

In order to validate our protocol and to assess the sensitivity of the method, a serial dilution of 1 µg of DNA from fungal pure cultures of *F. poae* were added to 0.2 g of fast food samples and DNA was extracted; the amplification protocol described above was then applied for the detection of DNA of trichothecene-producing *Fusarium* spp. from fast food samples in their original condition as well as from samples spiked with the DNA of trichothecene-producing *F. poae*. Furthermore, repeatability, measured as both the amount of

agreement between replicates within the same experiment and between replicates tested in different experiments, was determined testing multiple food samples in three experiments performed on separate days.

Results

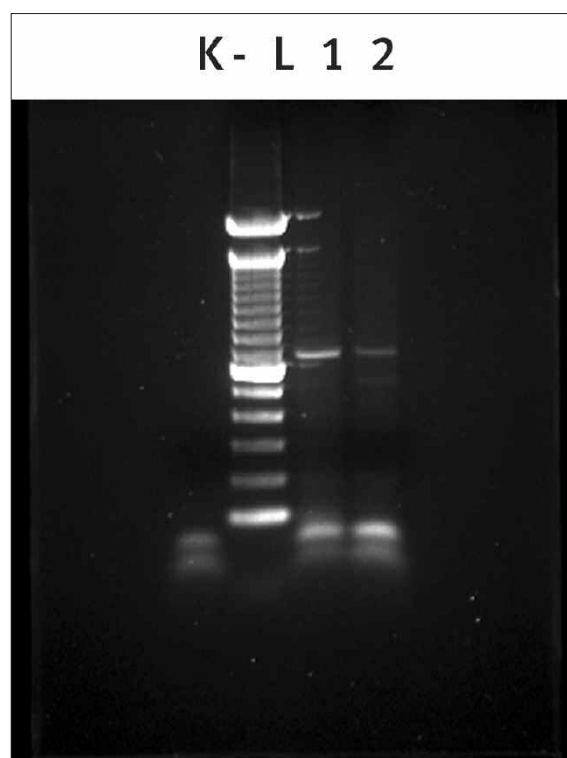
PCR detection of genomic DNA of trichothecene-producing *Fusarium* species in pure fungal cultures

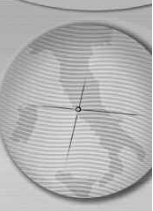
Using the PCR protocol described above, all genomic DNA extracted from trichothecene-producing *Fusarium* species in pure fungal cultures resulted in amplification of a 650-bp fragment which corresponded to the theoretical length of 658 bp deduced from the *tri5* gene sequence of *F. graminearum*. No amplicon was obtained when the DNA obtained from *F. avenaceum* and *E. tricinatum* were used as targets in the PCR experiments (Figure 1).

Besides, the two species-specific primer pairs only correctly amplified the respective DNA target from *F. graminearum* and *F. culmorum* (data not shown). No signal was obtained after PCR when DNA purified from the other reference fungi was used.

Figure 1. PCR detection of genomic DNA of trichothecene-producing *Fusarium* species in fast food samples.

K-: negative control; L: 100 bp ladder; 1: fast food samples spiked with DNA of trichothecene-producing *F. poae*; 2: naturally contaminated fast food samples.





PCR detection of genomic DNA of trichothecene-producing *Fusarium* species in fast food samples

When the amplification protocol was applied for the detection of DNA of trichothecene-producing *Fusarium* spp. from fast food samples spiked with DNA of trichothecene-producing *F. poae*, a 650-bp fragment was obtained as expected from the *tri5* gene sequence; the detection limit was 1×10^{-4} μg of DNA, thus defining the minimum detectable amount in contaminated food samples to be 50 ng/g. No amplicon was obtained from "Prontocone" in its original condition, while a 650-bp band was obtained from "Prontocrepe", from the naturally contaminated conditions, although the intensity of the produced fluorescent band was weaker than that one from the spiked specimen. No band was obtained from the negative control. All food samples tested gave highly consistent results in repeatability assays, thus demonstrating the within-lab and within/between-day precision of the method.

Experiments performed with species-specific primer pairs on naturally contaminated *tri5* positive samples showed a positive amplicon of 898 bp corresponding to the *F. graminearum* specific sequence.

Discussion

Contamination of cereal products with mycotoxins represents a significant hazard to consumer health and thus has received increasing attention from food safety authorities and legislators.[17] Fungal contamination may be detected by microbiological methods, able to identify viable mycelia in cultures, and thus reflecting the current contamination status. Chemical methods for the analysis of mycotoxins are highly sophisticated procedures requiring skilled personnel. A comprehensive review of analytical methods was published by Krska et al.[18] To assess the risk of transmission of trichothecene mycotoxins into the food chain, the food industry needs improved and simpler methods to maintain high quality standards in cereal products. One such improvement is the application of rapid methods based on PCR which can analyse the potential contamination of a sample with *Fusarium* species producing trichothecenes.[10] The quality of template DNA is the crucial factor influencing amplification efficiency, and has been addressed in a recently developed protocol for cereals and malt.[13]

The production activity of the emerging Italian food industry often focuses on the dietary needs of modern life i.e. quick-ready made meals and

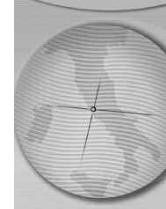
long-life products that maintain taste and quality. The aim of our project was to contribute to the quality assessment of those food types through the specific adaptation and validation of the protocol quoted above.

The most important *Fusarium* species such as *E.graminearum* and *F.culmorum*, which occur as trichothecene producers in all temperate regions worldwide, were targeted in the assay set up in this study. Results from the present study show that DNA from the reference *Fusarium* species and from the fast food samples can be rapidly identified by PCR. Furthermore, the potential for toxin production can be assessed in pure fungal cultures and in fast food samples. Our in-house validation study demonstrated sensitivity in the range previously shown for the original protocol [15] and to be repeatable for spiked and naturally contaminated food samples.

Thus, the method opens new opportunities to analyse DNA from trichothecene-producing fungi as an early control of raw materials and as a tool for quality assessment. Based on risk-assessment studies [19] in Central and Northern European countries, the European Commission has also expressed its opinion on trichothecenes as a source of danger to humans and animals, [20,21] therefore many countries in the world, along with the European Union, will probably have regulations for trichothecenes within the next few years. In order to meet these regulations the food industry needs a rapid and sensitive identification method for fungi relevant to the toxicological quality of its products. The sample preparation protocol described in this study, obtained by adapting published protocols, together with the use of primers of appropriate specificity in PCR assays, provide all of the features necessary for screening on an industrial scale over a relatively short time period, leaving only those samples that show the presence of toxicological potential, as identified by their PCR signal, needing exact chemical analysis. Information on the epidemiology of trichothecene producing *Fusarium* through the food chain and the identification of most frequently contaminated components of fast food are essential to develop effective public health strategies for minimising consumer exposure to trichothecenes and other mycotoxins.

Acknowledgments

This work was supported by grants from University of Catania - Progetti di Ricerca di Ateneo to A.A. and from Assessorato Agricoltura e Foreste - Regione Siciliana to S.S.



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