Identification of *Fusarium* spp. in hen's eggs

Ivana Regecová, Soňa Demjanová, Pavlina Jevinová, Monika Pipová

Institute of Meat Hygiene and Technology
Department of Food Hygiene and Technology
University of Veterinary Medicine and Pharmacy in Košice
Košice, Slovakia

Abstract

The development of detection and quantification systems for *Fusarium* spp. is time-consuming. It is, therefore, necessary to develop specific detection methods for this genus to facilitate monitoring of the environment and food for the presence of *Fusarium* spp. as a potential toxinogenic agent in food. The aim of this work was to find the most effective pre-isolation against self-isolation of DNA from food micromycetes, and then to identify *Fusarium* spp. at the genus level. By measuring the concentration and purity of the extracted DNA, isolation with pre-isolation, which involved the action of proteinase K and ultrasound on cells of the micromycetes examined, was demonstrated to be most effective.

DNA isolation, PCR, hen's eggs, Fusarium spp.

Introduction

The principal genera responsible for mycotoxins found in the human food chain are *Aspergillus*, *Penicillium* and *Fusarium*. Certain species of *Fusarium* are pathogens that may produce mycotoxins in infected material. They may also produce mycotoxins in plant and animal production under unfavourable storage conditions (Edwards et al. 2002). *Fusarium* mycotoxins are found primarily in cereal grain, in which the predominant mycotoxins are trichothecenes, moniliformin, fumonisins and zearalenone. Many toxigenic species of *Fusarium* are also associated with stored infected hen's eggs. Even with expert knowledge, macroscopic and microscopic identification is, however, particularly diversified in the genus *Fusarium* (D'Mello et al. 1999).

The development of molecular techniques has enabled more effective identification of this genus of micromycetes in samples of food (Kurtzman et al. 2011). One of these methods independent of cultivation is the polymerase chain reaction (PCR), which can quickly reveal specific sequences of micromycetes in food samples (Fredricks et al. 2005). PCR is widely used for the identification of genera and species of micromycetes. Special primers and sequencing methods are required for the cross-checking of morphological properties (Geiser et al. 2004). More frequent specific target sequences make it possible to determine the properties of a strain, which may be useful for its characterisation (Russell and Paterson 2006).

Determination of genera and species may also be used as a tool for the identification of potential toxinogenic risks associated with the presence of species capable of producing toxic compounds. PCR is, therefore, essential to preventative approaches in the area of food safety. However, the final sensitivity of any PCR test in detecting fungal pathogens is dependent on the effective lysis of cells of micromycetes and the purification of DNA that does not contain PCR inhibitors. Micromycetes, including the genus *Fusarium* spp., have cell walls that prevent lysis and the adequate isolation of nucleic acids. Only a few studies have focused on the critical phase of pre-isolation and extraction of DNA during the processing of food samples. In addition, highly sensitive and specific methods based on nucleic acids for the detection of micromycetes demand the use of DNA extraction agents that do not contain contaminating fungal nucleic acids (Pallez et al. 2014).

Address for correspondence:
DVM., PhD., Ivana Regecová
University of Veterinary Medicine and Pharmacy in Košice
Department of Food Hygiene and Technology
Institute of Meat Hygiene and Technology
Komenského 73, 041 81 Košice, Slovakia
E-mail: Ivana.Regecova@uvlf.sk
Phone: +421 918 997 630www.maso-international.cz

Materials and Methods

Micromycetes were isolated from 15 hen's eggs in accordance with the instructions given in STN ISO 21527-1. Subsequent macroscopic and microscopic examination confirmed the morphological features characteristic of the genus *Fusarium* spp. DNA was isolated from isolates initially identified as *Fusarium* spp.

Pre-isolation of DNA

Pre-isolation was performed in micro test tubes in two different ways:

1. By the addition of Proteinase K (Macherey-Nagel, Germany) and incubation for 30 min at 37 °C, and the action of liquid nitrogen for 5 min with subsequent heating at 95 °C / 5 min, repeated three times.
2. By the addition of Proteinase K (Macherey-Nagel, Germany) and zirconium beads (diameter 1 mm) to the samples and incubation for 30 min at 37 °C. The samples were then subjected to the action of ultrasound waves of 50 Hz for 10 min.

Isolation

Performed with an E.Z.N.A.® Fungal DNA Mini Kit (Omega Bio-Tek, USA). The DNA obtained in this way was subjected to the measurement of purity and concentration on a NanoDrop (Thermo Fisher Scientific, USA).

Identification of *Fusarium* spp. by PCR

Isolates of micromycetes were identified by a PCR method according to Abd-Elsalam et al. (2003) with the use of FIREpol® Master Mix (Ecoli s.r.o., Slovakia). A *Fusarium oxysporum* CCM F-485 reference strain was used as a positive control for PCR. The amplified PCR product (550 bp) at a quantity of 5 µl was analysed in 2.5 % agarose gel in TBE (Tris-borate-EDTA) buffer solution. Gel™ Red (Biotium Inc., USA) was added to the agarose gels for the visualisation of DNA. The individual products were visualised with a Mini Bis Pro® reader (DNR Bio-Imaging Systems Ltd., Israel). The PCR products obtained were subjected to sequencing (GATC Biotech AG, Germany) and the individual sequences were then compared with sequences in the NCBI GenBank database.

Results and Discussion

A total of 12 isolates of micromycetes were obtained by cultivation microbiological investigation of the shells of hen's eggs. These isolates were assigned to the genus *Fusarium* spp. by macroscopic and microscopic examination of micromycetes.

The isolates were then subjected to DNA extraction according to two different methods. Measurement of the concentration and purity of the isolated DNA showed isolation with pre-isolation, which included a mechanical action (zirconium beads, ultrasound) and the action of proteinase K on the cells of micromycetes, to be the most effective. All the DNA templates showed the highest concentration of DNA in the sample, ranging from 60 $ng \cdot \mu l^{-1}$ to 100 $ng \cdot \mu l^{-1}$, with this method. The purity of the DNA, which ranged from 1.8 to 2.1, also indicates that pre-isolation by means of a combination of a chemical and mechanical action is more effective. The second method, which employed the action of liquid nitrogen and proteinase K, proved inadequate for the isolation of DNA from *Fusarium* spp. cells. The isolated DNA was at low concentrations (> 9.0 $ng \cdot \mu l^{-1}$), and values for the purity of the DNA were beneath 1.600.

The measured values of concentration and purity confirmed the combination of mechanical and chemical action on *Fusarium* spp. cells to be the most effective pre-isolation. This assertion is also confirmed by the study by Pallez et al. (2014) who isolated DNA from *Fusarium* spp. micromycetes by means of a combination of mechanical action (microwave radiation) and chemical action (DNeasy Plant Mini Kit extraction; Qiagen, Hilden, Germany).

Identification of individual isolates by robust PCR (Fig. 1) was then performed, on the basis of which the genus *Fusarium* (20 isolates) was determined. Our results correlate with the study by Neamatallah et al. (2010), in which they demonstrated that 38% of tested eggs were contaminated with potentially toxinogenic micromycetes, of which 1% were micromycetes of the genus *Fusarium* spp.

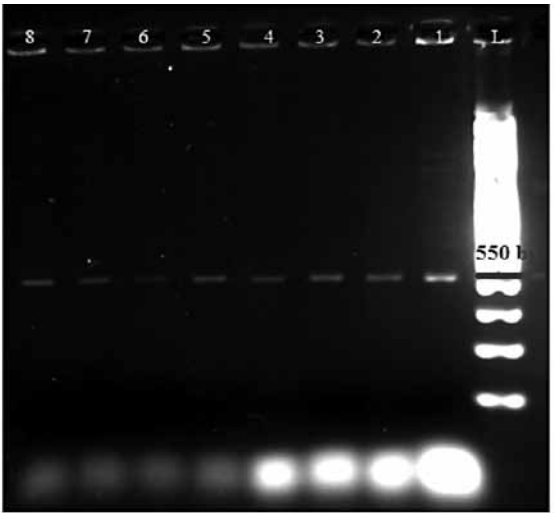

Fig. 1. Detection of a specific sequence of *Fusarium* spp. in 5.8S subunit rDNA - 550 bp (Regecová)

Conclusions

Current routine methods for the detection and identification of micromycetes require morphological and physiological characterisation, which is relatively time-consuming. The results of our work point to the possibility of identifying *Fusarium* spp. isolated from hen's eggs more effectively and more quickly.

Acknowledgements

This work was supported by IGA UVLF No. 01/2017 "Identification of toxigenic species of micromycetes isolated from eggs" and the Scientific Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic and the Slovak Academy of Sciences (VEGA 1/0705/16).

References

Abd-Elsalam KA, Aly IN, Abdel-Satar MA, Khalil MS, Verreet JA 2003: PCR identification of *Fusarium* genus based on nuclear ribosomal-DNA sequence data. Afr J Biotechnol **2**: 82-85

D'Mello JPF, Placinta CM, Macdonald AMC 1999: Fusarium mycotoxins: a review of global implications for animal health, welfare and productivity. Anim Feed Sci Technol **80**: 183-205

Edwards SG, O'Callaghan J, Dobson AWD 2002: PCR-based detection and quantification of mycotoxigenic fungi. Mycol Res **106**:1005-1025

Fredricks DN, Smith C, Meier, A 2005: Comparison of six DNA extraction methods for recovery of fungal DNA as assessed by quantitative PCR. J Clin Microbiol **43**: 5122-5128

Geiser DM, Jiménez-Gasco DS, Kang S, Makalowska NKI, Ward JT, Kuldau AG, O´Donnell K 2004: *FUSARIUM*-ID v. 1.0: A DNA sequence database for identifying *Fusarium*. Eur J Plant Pathol **110**: 473-479

Kurtzman CP, Fell JW, Boekhout T 2011: The yeasts, a taxonomic study. Elsevier, Burlington, USA, 137-144

Neamatallah AA, El-Leboudy A, Amer AA, El-Shenawy NM 2010: Biosafety against fungal contamination of hen's eggs and mycotoxins producing species. Met, Env & Arid Land Agric Sci **20**: 63-73

Pallez M, Pasquali M, Bohn T, Hoffmann L, Beyer M 2014: Validation of a Quick PCR Method Suitable for Direct Sequencing: Identification of *Fusarium* Fungal Species and Chemotypes for Preventive Approaches in Food Safety. Food Technol Biotechnol **52**: 351-358

Russell R and Paterson M 2006: Identification and quantification of mycotoxigenic fungi by PCR. Process Biochem **41**: 1467-1474

STN ISO 21527-1: Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of yeasts and moulds. Part 1: Colony count technique in products with water activity greater than 0.95