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Contamination with storage fungi of human food from Cameroon

Patrick Berka Njobeh [a,*], Mike Francis Dutton [a], Susan Hermina Koch [b], Anil Chuturgoon [c], Stoycho Stoev [d], Keith Seifert [e]

[a] Food, Environment and Health Research Group, Faculty of Health Science, 2028, University of Johannesburg, Doornfontein Campus, 2028 Gauteng, South Africa
[b] Plant Protection Research Institute, Agricultural Research Council, Private Bag X134, Pretoria 0001, South Africa
[c] Medical Biochemistry, School of Medical Sciences, Faculty of Health Sciences, Nelson R Mandela School of Medicine, University of Kwazulu-Natal, Private Bag 7, Congella, 4013 Durban, Kwazulu-Natal Province, South Africa
[d] Department of General and Clinical Pathology, Faculty of Veterinary Medicine, Trakia University, Student Campus, 6000 Stara Zagora, Bulgaria
[e] Biodiversity, Agriculture and Agri-Foods, 960 Carling Avenue, Ottawa, Ontario/Ottawa, Canada

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ABSTRACT

In a mycological study, a total of 95 human food samples were investigated to evaluate the incidence of fungal contamination in Cameroon by conventional identification method and partly confirmed by DNA sequencing. The isolated fungal spp. were further studied to determine their toxigenic potentials. The investigation revealed the predominance of *Aspergillus* and *Penicillium* with 96% of samples contaminated with at least one species of these fungi, whereas the incidence of co-contamination of samples was 85%. *Aspergillus flavus* and *Aspergillus parasiticus* (Flavi section) were the most predominant species contaminating mainly maize and peanuts. In addition, *P. crustosum* and *P. polonicum* were the most common contaminants belonging to the genus *Penicillium*. On the other hand, *A. ochraceus* (Circumdati section) registered a low incidence rate of 5%, including other members of the *Aspergillus* group. Other members of the genera *Rhizopus* and *Alternaria* spp. were also registered in the study. A majority of fungal strains of *A. ochraceus*, *A. parasiticus*, *P. crustosum* and *P. polonicum* isolated were toxigenic, producing the mycotoxins tested for, while none was detected in cultures of *A. fumigatus*. The high incidence rate of fungi contamination coupled with their potentials in producing mycotoxins gives a strong indication that the samples tested may likely be contaminated with various mycotoxins. There is need for further study to assess the incidence of mycotoxins contamination in similar food samples.

1. Introduction

Fungi are the major contaminants of foods in the world. Invasion of food by various fungi according to Sibanda et al. (1997) and Boysen et al. (2000) may result in remarkable rapid quality deterioration. As a consequence of this loss in quality, the profitability and effectiveness of food utilization are considerably reduced. Fungi are not only considered as significant plant pathogens, because of a wide range of diseases they cause in plant materials, they are also the principal sources of a class of secondary metabolites generally termed mycotoxins. Various reports (e.g., IARC, 1993; Pittet, 1998; D'Mello et al., 1999; Bankole and Adebanjo, 2003) on the incidence of mycotoxins particularly aflatoxins, ochratoxins, fumonisins and deoxynivalenol in cereals and cereal-based products are available.

The significant health risk posed in the consumption of mycotoxin-contaminated food products by both animal and man is of utmost concern (Danicke, 2002; D'Mello, 2003) in many developing countries. Studies on fungal and mycotoxin contamination in food commodities in Cameroon (Cardwell, 1999; Ngoko, 1999; Ngoko et al., 2001, 2002; Tagne et al., 2003; Ngoko et al., 2005) are limited with inadequate and not well documented data on this subject. Furthermore, to ensure a healthy food supply thereby minimizing consequences to consumers' health, there is a need to monitor fungal contamination periodically so as to meet the requirements of new legislations that are continuously being revised (Mashinini and Dutton, 2006). The present study therefore investigates food quality with respect to microbial contamination and their potential in producing mycotoxins in food products collected from various areas in Cameroon. The fungi from *Aspergillus* and *Penicillium* groups are widespread in nature and commonly contaminate stored grains in contrast of *Fusarium* spp., which are classified mainly as field fungi (Pitt and Hocking, 1997). In addition, the former two genera are able to grow at low moisture levels, unlike *Fusarium* spp., which require higher moisture levels for their growth. Therefore, the study on the incidence of *Fusarium* contamination in similar samples will be reported elsewhere.

2. Materials and methods

2.1. Sampling

A total of 95 dried Cameroonian samples of maize (*Zea mays* L), peanut (*Arachis hypogaea* L), bean (*Phaseolus vulgaris* L), soybean

* Corresponding author. Tel.: +27 11 559 6803; fax: +27 11 559 6227.
E-mail address: pnjobeh@uj.ac.za (P.B. Njobeh).

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(*Glycine max* Merr) and others including rice (*Oryza sativa*), cassava (*Manihot esculenta*) flakes and flour and pumpkin seeds (*Curcubita pepo*), intended for human consumption were randomly collected from separate batches through purchases and donations between December 2004 and April 2005 in the Cameroon. Samples were collected by thoroughly mixing the content from several points in the bag (top, middle and bottom). The samples (about 0.5 kg each) were categorized into two geographical regions viz.; western highlands (Kumbo and Bamenda) and tropical rainforests (Douala and Yaounde). Samples from Yaounde were collected in 2005. These samples were put in sealed plastic bottles and sent to South Africa, where they were immediately stored at −20 °C until analysis.

2.2. Fungi isolation

This involved four procedures: fungal isolation by placing a 1 ml of serially diluted 1 g blended material suspended in 9 ml ringer's solution on potato dextrose agar (PDA) and Ohio Agricultural Experimental Station Agar (OAESA) according to Kaufman et al. (1963); sub-culturing of isolated colonies on PDA, malt extract agar (MEA) and Czapek yeast extract agar (CYA) (See Appendix 1 for agar preparation procedures); followed by macro- and microscopic identification, while the final step involved DNA sequencing in case where identification by conventional method was not possible. Determination of each species of fungi was done using the keys of Klich and Pitt (1988) and Klich (2002) for *Aspergillus* spp. and Pitt and Hocking (1997) for *Penicillium* and other genera. This was done by observing both macroscopic characteristics of the colonies on various media used as well as the microscopic morphology and measurements of the conidiophores (after staining mycelia with 0.1% fuchsin dissolved in lactic acid) under an Olympus B061 Compound microscope (Wirsam Scientific, S. Africa) and Microscope Standard 19 (470919-9902/06) equipped with an Axiocam MRC Camera Ser. No. 2 08 06 0245 and AxioVision Release 4.5 SP1 (03/2006) software (Zeiss, West Germany).

2.3. DNA extraction, PCR and nucleotide sequencing

In a case where the morphological characteristics of individual fungal spp. were not sufficient for clear identification and depending on the relative importance of the fungus with respect to its potential to produce various mycotoxins, further analysis was performed. The technique involving the comparison of nucleic acid profiles of individual fungal species was, therefore, employed using a GeneAmp PCR System 9700 and an automated sequencer—ABI PRISM 3700 Genetic analyzer according to Samson et al. (2004).

Penicillium isolates for DNA sequencing were diluted and PDA was used to subculture isolates for 7 days at 25 °C. The mycelia were then scrapped and transferred into a 0.5 ml sterile screw-cap vial containing 200 µl of ringer solution, freeze-dried and stored at −40 °C until analyzed. Identification by DNA sequencing was conducted partly at the Biodiversity, Agriculture and Agri-Foods, Ontario, Canada and Inqaba Biotechnological Industries, (Pty) Ltd, South Africa. The frozen samples were kept at room temperature for about 3 h and genomic DNA extracted. Partial β-tubulin sequences were determined. A FastDNA® Kit (Bio101, Carlsbad, USA) was used to isolate the genomic DNA according to the manufacturer's recommendations. Bt2a and Bt2b primers were used to amplify the β-tubulin genes, while the PCR reactions were performed in 50 µl reaction mixture prepared by mixing 1 µl genomic DNA (10 ng/µl), 5 µl PCR buffer, 30 µl ultra pure sterile water, 10 µl dNTP (1 mM), 1 µl of each primer (50 pmol/µl) and 1 µl Taq polymerase (2.5U/µl DNA) (SpaeroQ, Leiden, The Netherlands). A GeneAmp PCR System 9700 (AB, Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands) was used in amplifying the β-tubulin template, programmed for 5 cycles of 1 min denaturation at 94 °C, followed by primer annealing for 90 s at 68 °C and extension for 2 min at 72 °C and drop in annealing temperature of 1 °C/cycle, followed by 25 cycles of denaturation at 94 °C for 1 min, followed by primer annealing for 90 s at 64 °C, extension for 2 min at 72 °C and a final 10 min elongation step at 72 °C. After amplification, a commercial GFX column, PCR DNA purification Kit (Amersham, Bioscience, Roosendaal, the Netherlands) was used to remove excess primers and dNTPs and purified PCR fragments were re-suspended in 50 µl 1× TE buffer (10 mM Tris–HCl, 1 mM Na_2EDTA, pH 8.0) containing RNAse A at 20 µg/ml. Samples were incubated at 37 °C for 30 min and then extracted with 300 µl of phenol/$CHCl_3$/isoamyl alcohol. The aqueous phase (~300 µl) was transferred to another tube and 25 µl of 7.5 M ammonium acetate and 125 µl of ethanol were added, mixed and incubated for 30 min at −20 °C. The samples were then centrifuged at 4 °C for 15 min at 12,000 ×*g* and purified PCR fragments were rinsed with 95% ethanol, air dried and re-suspended in 100 µl of 1× TE. Quality and quantity of the DNA obtained were determined by subjecting a portion of the preparation to agarose gel electrophoresis and UV spectrophotometry, respectively. A fraction of the DNA preparations were subjected to gel electrophoresis.

The primers were then used to sequence the PCR products in both directions with a DYEamic ET Terminator Cycle Sequencing Kit (Amersham, Bioscience, Roosendaal, The Netherlands). The cycle sequencing reaction mixture (10 µl) used consisted of 1 µl template DNA (10 ng/µl), 4 µl Dye terminator RR mix, 4 µl ultra pure sterile water and 1 µl primer (4 pmol/µl). The reactions were then run in a GeneAmp PCR System 9700 run in 9600 mode (AB, Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands), previously programmed for 25 cycles of 10 s denaturation at 96 °C, followed by primer annealing for 5 s at 50 °C and extension for 4 min at 60 °C. Purification of sequencing products was done as recommended by the manufacturer with Sephadex G-50 superfine columns (Amersham, Bioscience, Roosendaal, the Netherlands) in a multiscreen HV plate (Millipore, Amsterdam, the Netherlands) and with MicroAmp Optical 96-well reaction plate (AB, Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands).

Samples were then analyzed on an ABI PRISM 3700 Genetic analyzer (AB, Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands). The forward and reverse sequences were assembled using the programmes SeqMan and EditSeq from the LaserGene package (DNAStar Inc. Madison, WI). Alignments of the partial β-tubulin gene sequences data were calculated using a software package BioNumerics (Applied Maths BVBA, Saint Martens-Latem, Belgium) and adjustments made manually with the aid of an eye to maximize homology.

Reference cultures were grown on potato carrot agar (PCA) slants at 25 °C in the dark and deposited at the National Mycological Herbarium (PREM) of the Agricultural Research Council—Plant Protection Research Institute Culture Collection Library, Pretoria, South Africa.

2.4. Determination of the mycotoxigenic potentials of isolates

The fungal species so isolated in the present study were further analyzed for their capability to produce the following mycotoxins: aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), ochratoxin A (OTA) and an uncharacterised metabolite (UM). The isolates were individually cultured on solid YES agar in Petri dishes and incubated at 25 °C for two to three weeks according to the method of Singh et al. (1991). A multi-mycotoxin screening technique was then employed whereby 5 g of isolate including the medium was plugged and dissolved in 10 ml of dichloromethane. This solution was further filtered, and the filtrate was placed in a screw-cap vial and dried under a stream of N_2 gas and stored at 4 °C until analyzed.

A two-dimensional thin layer chromatographic technique (TLC) devised by Patterson and Roberts (1979) was employed for the detection of mycotoxins. Into the vial containing the extract, 200 µl of dichloromethane (DCM) was added, vortexed and 20 µl of extract solution spotted about 10 mm from the edge of a silica gel TLC plate. A similar procedure was followed for the standard mycotoxins. The