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Ochratoxin A and citrinin producing species of the genus *Penicillium* from feedstuffs

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ABSTRACT

In Spain, low ochratoxin A (OTA) levels have been detected in several pork products but there is no information published about the fungi involved in this OTA contamination. It is well known that *P. verrucosum* is much more frequently found on cereals in countries where they occasionally have OTA problems as in North European countries compared with South Europe where levels of OTA generally seem to be lower or not detected. Much less information is available about citrinin (CIT) and CIT producing species in cereals and their by products. The aim of this study was to determine, identify and characterize the occurrence of potential OTA and CIT producing *Penicillium* spp. from mixed feeds and raw materials purchased in the Spanish market and used as feedstuffs. A total of 155 *Penicillium* spp. isolates belonging to 34 species were analyzed in order to know if they are able to produce OTA and/or CIT. From these isolates, 11 *P. verrucosum* which were characterized by RAPD analyses, produced OTA. Fourteen isolates were CIT producers, 10 isolates of *P. verrucosum* and 4 of *P. citrinum*. Although the occurrence and abundance of OTA and CIT *Penicillium* producing species have been low in our study, our results confirm the potential risk of OTA and CIT production in feeds if stored improperly. Our results also confirm the occurrence of *P. verrucosum* in South European countries and that it is the only OTA producing *Penicillium* species in these substrates.

1. Introduction

Ochratoxin A (OTA) is a well-know nephrotoxic mycotoxin, which has also carcinogenic, teratogenic and immunotoxic properties. Natural occurrence of OTA has been reported from temperate to tropical climates in different food commodities such as cereals, coffee, dried fruits, wine and grape juice. In Europe, OTA maximum levels have been established for most of these foodstuffs (Commission Regulation (EC) No 123/2005). Some guidance values for this mycotoxin have been recently recommended for cereal, cereal products intended for animal feed, and complete and complementary feedingstuffs for pigs and poultry (Commission Recommendation (EC) No 576/2006).

Pigs are generally considered to be the most sensitive farm animal species to the nephrotoxicity of OTA. Chickens are also sensitive species, and it is assumed that this mycotoxin is the most cause of poultry nephropathy. Herbivores such horses and other species that rely on caecal rather than ruminal fermentation may absorb intact OTA in the small intestine being, probably, more sensitive than ruminants, but quantitative data are lacking (EFSA, 2004).

Contamination of animal feeds with OTA may result in the presence of residues in edible offal and blood products, whereas the OTA contamination in meat, milk and eggs is negligible. However, higher concentrations of OTA may occur in certain local specialties such as blood puddings and sausages prepared with pig blood serum (EFSA, 2004). At present, maximum levels for OTA in meat and meat products have been not established in the European Community. However, the consideration of setting a maximum level for OTA for edible offal and blood products is under discussion. In Denmark, since 1978, the contamination of pig meat with OTA has been assessed indirectly by the inspection of pigs' kidneys for the presence of macroscopic lesions of porcine nephropathy (Jorgensen and Petersen, 2002). Nephritis is a common cause of condemnation of pig kidneys in Great Britain, but there are few studies of OTA in cases of porcine nephropathy identified at slaughter in other countries (Gresham et al., 2006). In France, the first national monitoring programme showed that pigs are clearly exposed to OTA and monitoring of pork products and of feed for swine is necessary. Swine, like poultry, are exposed to OTA through their feed which is composed of cereals such as barley, maize, oats, wheat that are susceptible to contamination by this mycotoxin (Dragacci et al., 1999). In Spain, low OTA levels have been detected in several pork kidney samples (Canela et al., 1994) and in pig liver-derived pates (Jimenez et al., 2001) but there is no information published about the fungi involved in this OTA contamination.

Much less information is available about citrinin (CIT). It often co-occurs with ochratoxin A and has been implicated in mycotoxic nephropathy of pigs and in avian nephropathies. In cereals, it appears to be less important concerning cases of porcine nephrotoxicity in temperate regions than OTA (Frisvad and Thrane, 2000). Nephrotoxicity

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has been observed in other domestic animals such as dogs, rabbits and cattle. Citrinin is also embryotoxic, teratogenic and genotoxic (Abramson, 1997; Scott, 2004). Information on its mechanism of toxicity is limited and there is only limited evidence for the carcinogenicity of citrinin to experimental animals. At present, CIT is not subject to regulatory action (FAO, 2004). The main CIT producing species in foods are *Penicillium citrinum*, *Penicillium expansum*, *Pencillium verrucosum*, *Aspergillus terreus* and *Monascus ruber* (Cabañes et al., 2007).

Several species of the genera *Penicillium* and *Aspergillus* are known to form OTA (Cabañes and Bragulat, 2008). There is a long list of species known to produce OTA in the genus *Aspergillus*, but few of them are known to contaminate foods with this mycotoxin. OTA contamination of food and feeds was until recently believed to be produced only by *A. ochraceus* (=*A. westerdijkiae*) and by *P. verrucosum*, which affect mainly dried stored foods and cereals respectively, in different regions of the world. Recently, *P. nordicum*, formed with some strains isolated mainly from fermented meat and cheese, split from the latter species (Larsen et al., 2001; Castellá et al., 2002). At present, these two species are the only OTA producers known and accepted in *Penicillium* (Frisvad et al., 2004).

It is generally assumed that *P. verrucosum* produces OTA in temperate and cold climates, whereas *A. ochraceus* is more commonly associated with warmer and tropical climates. However, recent surveys have clearly shown that some species belonging to the black aspergilli, including the *Aspergillus niger* aggregate and *Aspergillus carbonarius*, are sources of OTA in food commodities such as wine, grapes, dried vine fruits among others (Cabañes et al., 2002; Abarca et al., 2003, 2004). *Penicillium verrucosum* has been reported to produce citrinin. Because this fungus is the major producer of ochratoxin A in cereals such as wheat and barley, it is not surprising that both mycotoxins often occur together although citrinin is reported much less frequently. Commercial mixed feeds, contain mainly mixtures of home-grown cereals and imported commodities. Mycobiota of cereal grains, mixed feeds and other raw materials are well documented, but there only a few published reports about the occurrence of OTA and CIT producing fungi from mixed feeds. In a recent study, among the OTA producing *Aspergillus* spp., only *A. niger* and *A. ochraceus* were recovered from mixed feeds and component raw materials from Spain (Accensi et al., 2004). However, the high occurrence of other potential OTA producers from Brazilian poultry feeds such as *A. carbonarius, A. melleus* and *P. verrucosum* have been recently reported (Rosa et al., 2006).

The aim of this study was to determine, identify and characterize the occurrence of potential OTA and CIT producing *Penicillium* spp. from mixed feeds and raw materials purchased in the Spanish market and used in animal nutrition for pigs, poultry and horses.

2. Materials and methods

2.1. Samples

A total of 178 feed samples were analyzed: 90 samples of cereals (comprising corn (n=44), barley (n=29) and wheat n=17) and 88 mixed feeds (comprising pig feeds (n=58), horse feeds (n=15) and poultry feeds (n=15). The samples were obtained from local agricultural cooperatives and factories and were stored at 4 °C and analyzed the day after collection.

2.2. Mycobiota determination

Enumeration of fungal propagules was done on solid media using the surface-spread method. Serial dilutions in saline were made and 0.1 ml aliquots were inoculated onto three plates of malt extract agar (MEA) (Pitt and Hocking, 1997) supplemented with 100 ppm of chloramphenicol and 50 ppm of streptomycin and onto three plates of dichloran rose bengal chloramphenicol agar (DRBC) (Pitt and Hocking, 1997). Plates were incubated at 28 °C for 7 days. Plates with 10–100 colony forming units (cfu) were used for counting and the results were expressed as cfu per gram of sample (cfu/g). However, plates with less than 10 cfu at the lowest tested dilution (10^{-1}) were recorded. Colonies belonging to *Penicillium* spp. were transferred to slants to ensure precise counting and then to plates for identification to species level (Pitt and Hocking, 1997). These isolates were preserved at −80 °C for later studies. All the *Penicillium* isolates were three-point inoculated on MEA, Czapeck Yeast extract Agar (CYA), 25% Glycerol Nitrate agar (G25N), and checked for their capacity of growth at 5 and 37 °C in CYA. Isolates were also inoculated on Creatine Sucrose agar (CREA) (Frisvad, 1985), Creatine Sucrose Neutral agar (CSN) (Pitt, 1993), Yeast Extract Sucrose agar (YES) (Filtenborg et al., 1990) and in a broth containing urea as a sole nitrogen source (Bridge, 1985) as additional tests for characterizing these species. The use of the Ehrlich reagent to detect indole metabolites (Lund, 1995) was also performed from colonies developed on CYA.

2.3. RAPD characterization of P. verrucosum strains

Eleven isolates that were morphologically identified as *P. verrucosum* were characterized by RAPD (Castellá et al., 2002). Representative strains of *P. verrucosum* and *P. nordicum* were also included in this study. The strains are listed in Table 1. Fungal DNA was extracted as described by Accensi et al. (1999), with slight modifications. The strains were inoculated in 1.5 ml eppendorf tubes containing 500 µl of Sabouraud broth (2% glucose, w/v; 1% peptone, w/v) supplemented with chloramphenicol (1 mg/l) and incubated in an orbital shaker at 25 °C and 300 rpm during 36–48 h. Mycelium was recovered after centrifugation and washed with NaCl 0.9% (w/v), frozen in liquid nitrogen and ground to a fine powder with a pipette tip. The powder was kept for 1 h at 65 °C in 500 µl extraction buffer (Tris–HCl 50 mM, EDTA 50 mM, SDS 3% and 2-mercaptoethanol 1%). The lysate was extracted with phenol:chloroform:isoamyl alcohol (49,5:49,5:1), 3 M NaOAc and 1 M NaCl. DNA was recovered by isopropanol precipitation. The pellet was washed with 70% (v/v) ethanol, dried under vacuum and resuspended in TE buffer.

The RAPD analysis was carried out as described by Castellà et al. (2002). The isolated chromosomal DNA was used as template DNA for the RAPD-PCR reactions. The PCR reaction mixture was performed in a total volume of 25 µl containing 0.1–10 ng of DNA, 0.55 µM of primer ari1 (5′-TGCTTGGCACAGTTGGCTTC-3′), 0.1 mM dNTPs and 1 U of taq DNA polymerase in the buffer provided by the manufacturer (Applera Hispania, Spain). The reactions were conducted in a Perkin Elmer GeneAmp 2400 thermal cycler which was set to the following conditions: 95 °C, 5 min; 44 cycles consisting of 1 min at 95 °C,

Table 1
Collection number and origin of *P. verrucosum* and *P. nordicum* strains studied

Strain	Origin: source, country	Strain	Origin: source, country
P-105	Poultry feed, Spain	P-437	Barley, Spain
P-194	Corn, Spain	P-438	Barley, Spain
P-198	Pig feed, Spain	P-440	Barley, Spain
P-293	Wheat, Spain	BFE 487[a]	Lentils, Sweden
P-297	Barley, Spain	BFE 524[b]	Wheat, U.S.A.
P-346	Barley, Spain	IBT 12803[c]	Raw sausage, Italy
P-364	Horse feed, Spain	IMI 200.310[d]	Unknown
P-410	Barley, Spain		

Abbreviations: P-, Culture Collection of the Veterinary Mycology Group, Barcelona, Spain; BFE, Culture Collection of The Federal Research Centre for Nutrition, Karlsruhe, Germany; IBT, Culture Collection of Bio-Centrum-DTU, Lyngby, Denmark; IMI, International Mycological Institute, Surrey, Egham, UK.
[a] *P. nordicum* (Larsen et al., 2001); genetic group I (Castellà et al., 2002).
[b] *P. verrucosum* (Larsen et al., 2001); genetic group II (Castellà et al., 2002).
[c] *P. nordicum* (Larsen et al., 2001).
[d] Neotype strain of *P. verrucosum*.