A Survey of occurrence of toxogenic fungi and mycotoxins in pig feed samples-Use in evaluation of risk assessment

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Abstract

In order to assess of risk assessment, the aim of this paper was to provide good and detailed insight into the level of contamination of complete feedmixes intended for fattening swine from mycotoxin-producing fungi and mycotoxins (n=18). Isolation and quantitative enumeration of fungal propagules were done on solid media using the standard microbiological procedure. These plates were incubated the number of colonies was determined and thent on the basis of characteristic colonies and microscopic analysis was performed to identify genera and species of moulds. Isolates identified as *Aspergillus* and *Penicillium species* were subjected to molecular characterization of the presence of genes responsible for the synthesis of OTA (polyketide synthase gene-PKS). Total fungal counts (CFU/g) ranged from 0,5x105 do 4x106. From a total samples analysed, seven samples had fungal counts higher than the limit established by Serbian regulations (3x105). During a mycological analysis of complete feedmixes intended for fattening swine, a total of six genera and 14 species of moulds were identified of which the most frequent one was of the genus Penicillium (94,4%) while the moulds from Fusarium genere isolated in 55,5% and Paecilomyces in 44,4% of the samples from investigated localities. Other fungi from the genera Aspergillus (22%), Mycor (11,1%) and Alternaria (5,5%) were represented in a less amount. Polymerase chain reaction (PCR) is a set of 18 isolates of the DNA belonging to families Penicillium and Aspergillus. The sequences of PCR reaction products in three samples were compared with nucleotide sequences of genes for poliketid synthase (PKS) from Penicillium species and found that the samples possess PKS sequence. The traditional methods for identification of ochratoxin-producing fungi are time-consuming and labor-intensive. Rapid and specific detection of ochratoxin-producing fungi is important for ensuring microbiological quality and safety of feed and food.

Key words: Moulds, Mycotoxin, Feed Stuff, PCR, Risk assessment, Porcine, Occurrence, Toxogenic

Introduction

The contamination of agricultural commodities with fungi able to produce toxic metabolites is one of the main worldwide concerns. Discoloration, quality deterioration, reduction in commercial value and mycotoxin production has been linked to mouldy contaminated foods and feeds. It not only generates great economic losses [1], but also represents a threat to human and animal health, particularly through the synthesis of mycotoxins.

Mycotoxins are naturally occurring secondary metabolites of several toxigenic microfungi that contaminate the whole food chain, from the agricultural cultures to the plate of consumers. Mycotoxins occur sporadically both seasonally and geographically. In farm animals, mycotoxins exert their effects through four primary mechanisms: (1) intake reduction or feed refusal, (2) alteration in nutrient content of feed in nutrient absorption and metabolism, (3) effects on the endocrine and exocrine systems and (4) suppression of the immune system. In routine animal feed screening, mycotoxins are usually found at relatively low levels.

Limited information exists regarding the effects of low levels of multiple mycotoxins in livestock. It has been suggested that combinations of mycotoxins at

low concentrations may have negative effects on livestock, even though the concentrations of individual mycotoxins are well below concentrations reported to cause negative effects [2].

The main mycotoxins classes of concern produced by fungi in the genera Aspergillus, Penicillium and Fusarium include the aflatoxins, ochratoxin A, trichothecenes and fumonisins. Moisture and/or temperatures is the single most important factor in determining if and how rapidly molds will grow in feeds. Improper storage accompanied by too high a temperature and elevated moisture content in the grain favours further mycotoxin production and leads to reduction in grain quality [3,4]. Therefore, rapid and specific detection of mycotoxogenic moulds is important for ensuring both microbiological quality and safety of both feed and food. The currently employed methods for identification of foodborne molds require culture isolation and application of morphological and physiological tests [5,6], which are time-consuming, labor-intensive, and often require mycological expertise [7]. Further, plate count techniques do not detect dead fungi, which could indicate past contamination of a product [5]. Nucleic acid-based methods, such as the polymerase chain reaction (PCR) and DNA probes, provide powerful and rapid tools for the detection of microorganisms. Although PCR has been widely used for the detection of foodborne bacteria and viruses, its application for specific detection of foodborne molds is relatively limited.

The aims of this work were: 1) to determine the mycobiota in pig feed samples, 2) to develop and optimize a PCR for rapid and specific detection of ochratoxogenic moulds, 3) to evaluate the feedstuffs' mycotoxins contamination, especially aflatoxins, ochratoxin, deoxinivlenol and zearalenone, 3) Also, the objectives of this study were to determine the effect of the moisture content and water activity (aw), on the mycobiota presence versus specific mycotoxins contamination.

Material and methods

Samples: The research materials consisted of 18 representative pig feed samples which were collected directly at animal farms from different provinces of Serbia during six month period. The samples (each about 1 kg) were stored at 4 °C and analysed the day after collection. On the same day, the moisture content of the feeds samples was determined by drying.

Reagents: Standards of AFT, OTA, ZEA and DON were purchased from Sigma-Aldrich Chemie GmbH. All other solvents and reagents were analytical grade.

1. Isolation and identification of fungal

Culturable fungal spore concentrations are presented in terms of colony-forming units (CFU)/g of samples. Isolation and quantitative enumeration of fungal propagules were done on solid media using the surface- spread method by blending a 10 g portion of each sample with 90ml of 0.1% peptone water solution. Serial dilutions, 10-1 to 10-6 concentration, were made from each material and 0.1ml aliquots were inoculated in triplicate on two media potato dextrose agar (PDA) and Czapek yeast extract agar (CYA) of fungal enumeration. After 3-7 days, growing fungal colonies were transferred to Czapek yeast extract agar (CYA) and incubated at 25 ^{0}C in the dark for 7 days. Macrofungi and moulds were identified to genera/species by their macro- and micromorphology features using appropriate identification keys [8-12].

2. Mould identification by molecular methods

A molecular method was used in order to eliminate any uncertainty in mould identification based on the aforementioned traditional methods. Eighteen isolates were tested using both methods and were subsequently compared to determine accuracy.

2.1. Primer selection

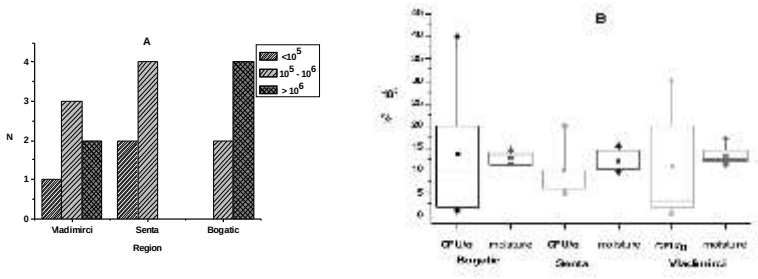
In this work, we used two sets of specific primers, AoLC35-12L/AoLC35-12R and AoOTAL/AoOTAR, from a 3.4 kb DNA sequence of a polyketide synthase (PKS) gene from A. ochraceus NRRL 3174. These primer sets were tested by PCR method on different genera of OTA and other mycotoxin producing fungi [13].

2.2. Fungal strains and culture conditions

Fungal strains identigfied as ochratoxogenic were grown at 25 ^{0}C on potato dextrose agar (PDA) (Difco, Fisher Labosi) during 7 days. Then spores were collected with a sterile solution of 0.1% (v/v) Tween 80 (Fisher Labosi) and stored at - 20 ^{0}C in 25% (v/v) of glycerol (Fisher Labosi) before use. Conidia (about 10^6/ml) were inoculated into 250-ml Erlenmeyer flasks containing 100 ml of potato dextrose broth (PDB) (Difco, Fisher Labosi), at 25 ^{0}C, without shaking for 3 days. The mycelium was harvested by filtration through a 0.45 Am filter (Millipore) frozen in liquid nitrogen and then stored at -80 ^{0}C before nucleic acid extraction.

2.3. Genomic DNA extraction

200 mg of lyophilized mycelium was homogenized in 800 Al of lysis buffer (100 mM Tris–HCl pH 7.4 (Sigma Aldrich), 20 mM EDTA (Sigma Aldrich), 250 mM NaCl (Sigma Aldrich), 2% w/v SDS (Sigma Aldrich)) by using a Ultra- Turax (Labo moderne) and incubated at 37 ^{0}C for 30 min with 10 Al of 25 mg/ml RNase solution (Promega), then added with 10 Al of proteinase K (20 mg/ml, Euromedex) were added and the mixture was incubated at 65 ^{0}C for 30 min. A volume of phenol-chloroform-isoamylic alcohol (v/v/v: 25/24/1) (Sigma Aldrich) was added, and the mixture was vigorously vortexed for 5 min. The aqueous phase collected after centrifugation (15,000 x g, 15 min) was extracted by an equal volume of chloroform (Prolabo). Genomic DNA was precipitated at - 20 ^{0}C in 2 h with two



Fugure 1. Total fungal counts (CFU/g) (A) and mean contamination levels of moulds and moisture content (B)

volumes of 100% ethanol (Prolabo). The DNA was pelleted by centrifuging at 15,000xg and washed with 1 ml of 75% ethanol then dried at the room temperature. 100 Al of water was used to re-suspend genomic DNA.

2.4. PCR reaction

PCR was performed with the Taq recombinant polymerase (Invitrogen, USA). Amplification was carried out in 50 Al reaction mixture containing: 5 Al of Taq polymerase buffer10X, 1.5 Al of 50 mM MgCl2, 1 Al of dNTP 10 mM of each (Promega), 1 AM of each primer, 1.5 U of Taq, about 50 ng of DNA genomic, H2O up to 50 Al. Reaction conditions were: 94 ^{0}C for 4 min (94 ^{0}C for 40 s, 58 ^{0}C for 40 s and 72 ^{0}C for 40 s) x 35 cycles followed by an incubation at 72 ^{0}C for 10 min. The amplified products were examined by agarose gel electrophoresis. The b-tubulin gene was used as positive control and primer sequences were:

TubF: ctcgagcgtatgaacgtctac;

TubR: aaaccctggaggcagtcgc,

which amplified a 340 bp fragment on genomic DNA.

2.4.1. Detection of PCR product

Gel electrophoresis was used to detect the presence of the amplified PCR product from each mold isolate. A volume of 20 Al of PCR products was subjected to electrophoresis on a 1.2% agarose gel; the gel was stained with ethidium bromide, and viewed under ultraviolet light to detect the presence and size of the amplified DNA product.

2.5 DNA sequencing

PCR products were purified to remove excess primer using micro concentrators, and then directly sequenced with the Dye-Deoxy Terminator Cycle Sequencing kit in an automated DNA Sequencer (BMR Genomics-Servizio Sequenziamento, Italy).

Chemical analysis

All pig feed samples were analysed with validated methods and under quality assurance conditions.

Moisture content

The moisture content of the pig feed samples was determined by drying at 105°C until the weight did not change further [14]. The aw value of the grain was measured in a hygroscope (GBX Scientific Instruments FA-St/1, tastatura model MX 3700/ML 4700) at a temperature of 20°C.

Analysis for aflatoxins (AFT), ochratoxin A (OTA) deoxynivalenol (DON) and zearalenone (ZEN). The detection of these mycotoxins in all samples were performed by TLC using appropriate methodology [15-18].

Statistical analysis

Differences in the mean levels of mycotoxins contamination across the three groups of positive samples was calculated by analysis of variance and then by a Student's t-test. Additional posttests were applied to evaluate differences between groups with statistically significant variation among means. The differences with p values smaller than 0.05 were considered statistically significant.

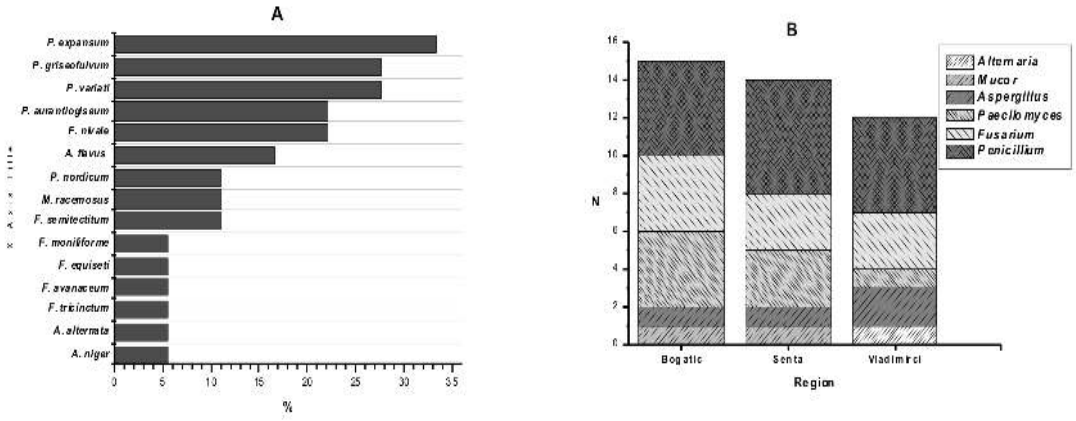
Results and Discussion

Mycoflora analysis

The results obtained from the mycoflora analysis in the samples are presented in Figure 1 and 2. Occurrence of mycotoxins contamination and mean contamination levels of mycotoxins (mg/kg) in samples originating from region where samples were collected are presented in Figure 4 and 5.

Total fungal counts ranged from 10^5 to $40x10^5$ cfu/g. In our study, the highest fungal count ($40x10^5$ cfu/g) and average total fungal counts was detected in pig feed samples collected from region Bogatic. From the 18 samples analysed, seven samples had cfu/g higher than the limit established by Serbian regulations ($3x10^5$) [19] Fungal count is an indicator of the quality of feeds and should not exceed $1x10^5$ cfu/g [20]. It is worth mentioning that in such a samples only Penicillium species mostly belonging to F. equiseti were isolated.

Species determination revealed great heterogeneity. A total of six genera and 14 species of moulds were identified. With the exception of Alternaria alternata, and Mucor racemosus which occurred only in one to two samples, the rest of the species were found in more than one sample in all locations surveyed. The most frequently isolated fungus was



Fugure 2. Relative frequencies of isolation of the dominant fungal species (A) and genus (B)

Penicillium species (Fig. 2), a mould present on 17 (94,4%) of the 18 samples examined, and comprised 30,6% ofthe total fungal population. Other frequently isolated moulds included *Fusarium* (55,5%) and *Paecilomyces* (44,4%) genera. *Fusarium* isolates were identified as *F. equseti, F. moniliforme, F. nivale F. semitectitum, F. tricinctum* and *F. avanaceum,* which are proven toxogenic moulds. Other fungi from the genera Aspergillus (22%), Mycor (11,1%) and Alternaria (5,5%) were represented in a less amount. Among fourteen species were identified, only *Paecilomyces* was not potentially mycotoxins produced fungi. The incidences of Penicillium species increased throughout the winter months whereas the Fusrium did not show any well defined pattern of occurrence. Penicillium occurrence in the mixed feed samples is in agreement with the results reported by other authors [21-24]. This shows that the incidence of these various species was important, as the produce was stored for prolonged periods of time.

PCR analysis

A total of six genera and 14 species of moulds were identified from the casing of different feed samples produced in a variety of production facilities of Serbia. The most frequently detected ochratoxogenic species were *P. expansum, P. griseofulvum, P. auatogriseum, P. nordicum,* and *Aspergillus flavus*. These species, except last one is the main species thought to be responsible for OTA production. However, this strain of *Aspergillus* was isolated at a significantly lower frequency.

The distribution of fingerprint profiles indicates that the most frequently occurring *Penicillium expansum* and Penicillium griseofulvum types are not factory-specific, i.e. the same profies of both species are found in the different factories which are geographically separated. To explain the dominance of some fingerprinting types there are at least three

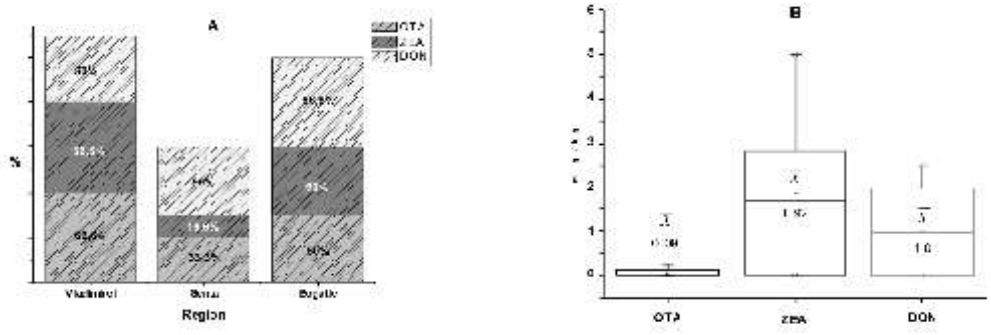


Figure 3. Occurrence of mycotoxins contamination in samples depending of regions (A) and mean contamination levels of mycotoxins (mg/kg) in samples originating from region Vladimirci (B)

Tabe 1. Fungal strains used and results of PCR conducted with AoLC35-12L/AoLC35-12R and AoOTAL/AoOTAR primers

Sr.No.	Isolate	The specificity of primer pairs		
		AoLC35-12F/AoLC35-12R		AoOTAL/ AoOTAR
1.	*Penicillium spp*		/	/
2.	*Penicillium expansum*		687 bp	/
3.	*Penicillium spp*		695 bp	/
4.	*Penicillium spp*		695 bp	/
5.	*Penicillium spp*		/	/
6.	*Penicillium spp*		/	/
7.	*Penicillium spp*		/	/
8.	*Aspergillus flavus*		/	/
9.	*Penicillium spp*		/	/
10.	*Penicillium spp*		/	/
11.	*Penicillium spp*		/	/
12.	*Penicillium spp*		/	/
13.	*Penicillium spp*		/	/
14.	*Penicillium spp*		/	/
15.	*Penicillium spp*		/	/
16.	*Penicillium spp*		/	/
17.	*Aspergillus ochraceus*. NRRL	3174	520 bp	/
18.	*Penicillium verrucosum* NRRL	3711	/	/

negative PCR response./

possibilities: 1) the dominant types may be linked to a genotype particularly well adapted to grow on feed, 2) the dominant profiles may bemore dominant in nature; 3) the dominant profiles match the ancestral profile while the less dominant fingerprinting profiles may be more recently derived. These three possible explanations are not mutually exclusive. The set primers AoOTAL/AoOTAR could be used to detect specifically A. ochraceus by PCR method, and AoLC35-12L/AoLC35-12R to detect OTA producing fungi, *A. carbonarius, A. melleus, A. ochraceus, A. sulfureus, P. verrucosum* and citrinin producing, *P. citrinum* and *Monascus ruber*. Further work is needed to validate their application to detect OTA producers on feedstuffs.

Relationships between the moisture content, the storage duration and the incidence of moulds isolated

During the collection period, the moisture content observed per pig feed samples varied between 9,63 and 17,3% (Fig. 1B). The moisture content incresed during period september-december, and reached in the december (17.25%), whereas decreased values at the end of the monitoring period 9,63%. However, mean moisture content observed per region was similar, and varied between 12,2% (Senta) to 13,36% Vladimirci. On the basis of obteined results can conclude, as the storage period was extended, the moisture content reached. Additionally, positive and significant correlations between the level of contamination of samples by total fungal counts and the moisture content were found in all of exmined regions (r=0.475). Fungal colonization, growth and sintesis of toxins, results from the complex interaction of several factors (water availability, temperature and incubation time) and therefore, an understanding of each factor involved are essential to understand the overall process and to predict fungal spoilage in agricultural and food products [3]. Improper storage accompanied by too high a temperature and elevated moisture content in the grain favours further mycotoxin production and leads to reduction in grain quality [25-27]. Isolated species in our case are mostly storage contaminators, implicating that the high number of contaminated feed is most probably the result of manipulative mistakes during storage of feedstuffs or feed.

Occurrence of mycotoxins in samples

The results obtained from the analysis of mycotoxins in the pig feed samples are presented in Figure 4 and 5. The predominant mycotoxin for all analyzed samples was DON, while, on the other hand aflatoxins was not detectable. The incidence of DON and OTA in all the samples was 55.5% and 50%, respectively, while the median content of the positive samples was 0.78 and 0.06 mg/kg. Therefore nine samples greatly exceeded the legally established DON limit (0.60 mg/kg) [20]. In respect to OTA content in only one sample from region Vladimirci OTA content (0.27 mg/kg) was exceeded the legally established OTA limit (0.25 mg/kg) [19]. The occurrence of ZEA was 44.5% and contamination levels fluctuated in very wide range between 0.20 mg/kg (mean 0,03 mg/kg) (Senta) and 5.0 mg/kg (mean 1,92 mg/kg) (Vladimirci). The incidence of ZEA was significantly different in samples originatig from Senta and Bogatic ($p< 0.001$). Six pig feed samples was contaminated with ZEA above the legally established ZEA limit (1.0 mg/kg). No significant

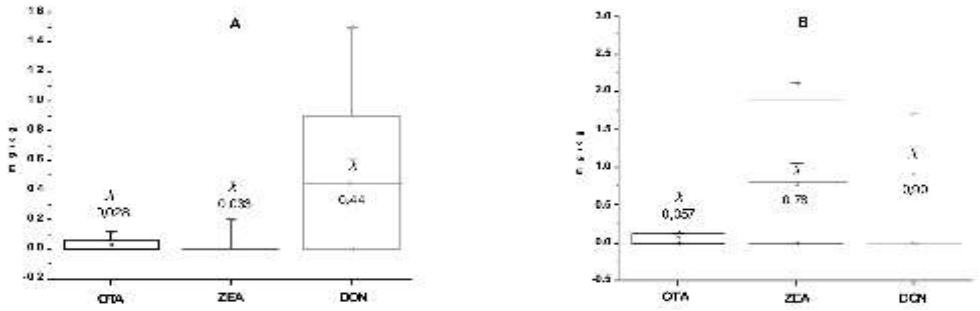


Figure 4. Mean contamination levels of mycotoxins (mg/kg) in samples originating from region Senta (A) and Bogatić (B), (ZEA $p < 0,001$).

differences were found between the median DON and OTA contents for all feed items ($p>0.05$). Additionally, positive and significant correlations between the level of contamination of samples by total fungal counts and the OTA content ($r=0.584$) were found, as well as the moisture content and the OTA content ($r=0.468$). In respect to correlations between the level of contamination of samples by mycotoxins, between OTA and ZEA content and ZEA and DON content, significant correlations were found $r=0.482$ and $r=0.358$, respectively.

These results are comparable with the data reported by other authors and with recently publisched data in Serbia [21-24,28], and Europe [39-31]. The results shown the natural co-occurrence of both toxins in majority of samples (55.5%) at minimal concentrations of 0.057 mg/kg (OTA) up-to 5.0 mg/kg (ZEA). Differences between the incidence and contaminations levels of mycotoxins in pig feed samples obteined in the present study may be attributed, among others, to a different origin of basing corn, it is well known that cereal infection with moulds and toxin production depend strongly on environmental conditions (damp climate, cool temperatures). However, these data must be interpreted with caution as they were calculated from a limited number of samples.

Conclusion

This study confirms the importance of continued surveillance of mycotoxigenic mould and mycotoxin occurrence in feeds in Serbia. More information is needed about why mycotoxins occur, when to expect them, how to prevent their occurrence and how to deal with their presence. A primary focus for continuing research is the development of management strategies to reduce the incidence of ochratoxogenic strains, in feedmixes. Serbian producerss will use these strategies to minimise the risk of mycotoxins development in grain and feedmixes. Combined data will enable us to realize the goal of developing realistic and accurate decision support systems for effective conservation of grain post-harvest. Improved screening techniques are needed for monitoring moulud and mycotoxin occurrence, diagnosing toxicities and prevention and treatment. The PCR could potentially be used as a rapid tool for screening both feed and food for the presence of ochratoxogenic strains..

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