Detection of aflatoxinogenic *Aspergillus* species in Bosnian sudzuk

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Abstract

The objective of the present study was to investigate the presence of *Aspergillus* species in Bosnian *sudžuk*, the most preferred dry sausage in Bosnia and Herzegovina, and evaluate their aflatoxigenic potential. A total of 145 bulk and vacuum packed samples of Bosnian *sudžuk* were collected from retail in the Sarajevo region; 105 samples from individual artisanal producers, and 40 samples were industrially produced Bosnian *sudžuk*. In total, four and seven *A. flavus* and *A. parasiticus* were isolated, respectively, while one or more genes responsible for biosynthesis of aflatoxins (*nor*-1, *ver*-1, *omt*-1 and *apa*-2) were detected in eight isolates. Aflatoxigenic strains were not found in the industrially produced samples. This is the first report about the presence of aflatoxigenic *Aspergillus* species in Bosnian *sudžuk*.

Key words: *A. flavus; A. parasiticus;* aflatoxigenicity; Bosnian *sudžuk*, aflatoxin genes

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Introduction

Foodstuffs, in general, present an ideal base for growth of filamentous fungi able to produce mycotoxins under favorable conditions. Over 64,000 moulds, yeasts and similar organisms were identified in the environment. On the other hand, only 114 mould and 12 yeast species are used in the food industry, out of which only 65 mould species have displayed ability to synthesize more than 150 different mycotoxins. In terms of meat and meat products, 78 species of moulds were isolated, and 50 of these are potentially toxigenic (Laciakova et al., 2004; Oswailer, 1996).

Aflatoxins, the secondary metabolites of *Aspergillus flavus* and *Aspergillus parasiticus*, are considered as the most potent hepatotoxins, hepatocarcinogens, mutagens and immunosupressors. Thus, prevention of their intake into a human organism through foods is considered of vital importance (Coppockand Christian, 2007; Frisvad et al., 2005; Levin, 2012).

In order to protect consumers' health, it is necessary to use rapid and reliable methods for selective detection of different species of fungi in foods of animal and plant origin. Contemporary mycotoxigenic mould analysis relies on PCR methods to determine target genes necessary for the biosynthetic pathway of mycotoxins (Paterson, 2006).

Previous studies have been conducted worldwide to determine the presence of aflatoxigenic species of moulds and their toxins in various foodstuffs. Bosnian *sudžuk* is a type of dry fermented sausage made out exclusively of beef meat and fat, seasoned with salt and pepper. Investigation of presence of aflatoxigenic moulds in dry meat products have not been undertaken in Bosnia and Herzegovina in the past. However, there are few reports focusing on their presence in foodstuffs similar to those traditionally produced and consumed in Bosnia and Herzegovina as well as the presence of aflatoxins in these products (Cvetnić and Pepeljnjak, 1995; Kivanç et al., 2006; Škrinjar and Horvat-Skenderović, 1989).

The objective of the present study was to investigate the presence of aflatoxigenic *Aspergillus* species in artisanal and industrially produced Bosnian *sudžuk,* and evaluate their aflatoxigenic potential

Material and Methods

Samples, isolation and identification of *Aspergillus* species

A total of 145 samples of Bosnian *sudžuk* were randomly collected from retail in the Sarajevo region; 105 (72 bulk and 33 vacuum packed) samples from individual artisanal producers, and 40 (21 bulk and 19 vacuum packed) samples of industrially produced Bosnian *sudžuk*.

Initial suspension and the first decimal dilution were prepared in accordance with standard method (ISO, 2003) using 10 g of each sample and purified peptone water. Following the preparation, 0.1 ml of initial suspension and the first dilution of the samples were streaked onto Sabouraud dextrose agar (SDA – Merck, Germany) and aerobically incubated at 25 °C for at least 5 days. In addition, the method of direct swab sampling of the sausage surface and plating on SDA (Samson et al., 2004) was also applied. Further subcultivation was performed

on SDA and Sabouraud dextrose broth (SDB – Merck, Germany). *In situ* appearance of conidia were examined under stereomicroscope (Leicka Zoom 2000™; Leicka Microsystems, UK) and septation of fungal hyphae using dark field microscopy (Olympus CX 41™; Olympus Life Science, Japan)(1).

Reference strains used

A. parasiticus ATCC 26864 and *A. flavus* ATCC 32592 were used as positive controls for routine species identification and PCR detection of aflatoxin genes in the isolates. These reference strains possess all four target genes (*nor*-1, *ver*-1, *omt*-1 and *apa*-2) necessary for biosynthesis of aflatoxins B_1 and G_1 (*A. parasiticus* and *A. flavus*), B_2 and G_2 (*A. parasiticus*) (3).

PCR detection of the aflatoxin-synthesizing genes

Preparation of *Aspergillus* strains for DNA extraction - Cultures of fungal isolates and reference strains were prepared using a modification of a previously described method (Chen et al., 2002) as follows: spore from SDA culture were inoculated with a swab into 0.5 ml of SDB and incubated at 25 °C±1 for 2 to 3 days. After incubation, mycelium was harvested by centrifugation at 8000 rpm for1 min and washed twice with sterile distilled water. Pellets were then dried with filter paper and heated for 15 minutes at 65 °C. After drying pellets were frozen in liquid nitrogen and then homogenized by crushing. Procedure of freezing and homogenization was repeated.

DNA extraction method - DNA extraction was performed following the procedure described by Cenis (1992). Briefly, 300 µL of extraction solution (200 mM Tris HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and 150 µL of 3 M sodium acetate (pH 5.2) were added to pellet, mixed and frozen at - 80 °C for 10 minutes, then defrosted and centrifuged for 5 minutes at 13200 rpm. The supernatant was then transferred to a new microtube with addition of the same amount of isopropanol, incubated for 5 minutes at room temperature, and centrifuged for 15 minutes at 13.200 rpm. The pellet obtained was washed with 300 µL of 70% of ethanol and again centrifuged. Ethanol was removed, DNA dried at 100 °C for 5 minutes and dissolved in 50 µl of TE buffer.

PCR and electrophoresis - PCR analysis was conducted according to Chen et al. (2002), with some modifications. PCR reaction mixes were made up in 25 µl volumes containing: 2.5 µl 10× PCR buffer (Genekam), 1.5 mM $MgCl_2$ (Qiagen), 100 µM of each dNTP (Sigma), 1.5 U DNA polymerase (Genekam), 1 µM primer (METABION international, AG) (Table 1), and 5 µl extracted DNA. The reaction was performed in a thermocycler (Applied Biosystems) with an initial DNA denaturation at 95 °C for 3 min, followed by 35 cycles of 95 °C 1 min; 65 °C 2 min, 72 °C 2 min and with a final extension at 72 °C 5 min. The PCR products were separated by capillary electrophoresis (QIAxcel Advanced apparatus), using QIAxcel DNA High-Resolution Kit, QX Alignment Marker (15 bp-10 kb) and QX DNA Size Marker 100 bp-2.5 kb (Qiagen).

Results

Aspergillus spp. was isolated from 11 of 145 tested samples (Table 2), and most of the isolates (n=10; 14.5%) were recovered from artisan *sudžuk*. *A. parasiticus* (n=7) was the most commonly identified species (63%), being detected in artisan *sudžuk*, whilst *A. flavus* (n=4 or 37%) was found both in artisan and industrial *sudžuk*.

Similarly, *A. parasiticus* showed a slight dominance in the overall number of aflatoxigenic genes because five of the eight isolates (62.5%) harboured one or more of the four aflatoxigenic genes, while *A. flavus* isolates were represented in 3 samples (Table 3).

Table 1. Primer pairs used in the PCR analysis (Chen et al., 2002)

Target gene	Primer design	Size of specific amplicon (bp)
***aflD* (nor-1)**	*nor*-1: 5’-ACC GCT ACG CCG GCA CTC TCG GCA C-3’ *nor*-2: 5’-GTT GGC CGC CAG CTT CGA CAC TCC G-3’	400
aflM (ver-1)	*ver-1*: 5’-GCC GCA GGC CGC GGA GAA AGT GGT-3’ *ver-2*: 5’-GGG GAT ATA CTC CCG CGA CAC AGC C-3’	538
aflP (omt-1)	*omt-208*: 5’-GGC CCG GTT CCT TGG CTC CTA AGC-3’ *omt-1232*: CGC CCC AGT GAG ACC CTT CCT CG-3’	1025
***aflR* (apa-2)**	*apa-450*: 5’-TAT CTC CCC CCG GGC ATC TCC CGG-3’ *apa-1482*: 5’-CCG TCA GAC AGC CAC TGG ACA CGG-3’	1032

Table 2. Distribution of *A. flavus* and *A.parasiticus* contamination of Bosnian *sudžuk* by packaging (bulk or vacuum) and production type (artisan or industrial) (n=145).

	Bulk artisanal sudžuk (n=72)**	Bulk industrial sudžuk (n=21)	Vacuum-packed artisanal sudžuk (n=33)	Vacuum-packed industrial sudžuk (n=19)
No. of *A. flavus and A.parasiticus* positive samples (%*)	4 (2.75)	1 (0.70)	6 (4.15)	0
Total No. (%)	5 (3.45)		6 (4.15)	

* - percentages of the total 145 samples; ** – number of sampled packaging of Bosnian *sudžuk* in each category

One or more of the four genes responsible for aflatoxin production were identified in eight of the 11 isolates (Table 3). One *A. flavus* isolate and two *A. Parasiticus* isolates did not harbour any of these genes. All the aflatoxigenic strains were isolated from the artisanal produced Bosnian *sudžuk*.

Table 3. Aflatoxigenic profile of *A. flavus* and *A. parasiticus* isolates from Bosnian *sudžuk*

Bulk or vacuum packed	Target genes *aflD* (*nor*-1)	*aflM* (*ver*-1)	*aflP* (*omt*-1)	*aflR* (*apa*-2)	Species
vacuum	+	+	+	+	*A. parasiticus*
vacuum	+	+	+	+	*A. flavus*
bulk	-	+	+	+	*A. parasiticus*
bulk	-	-	+	+	*A. flavus*
vacuum	+	-	-	+	*A. flavus*
vacuum	+	-	-	-	*A. parasiticus*
bulk	-	-	-	+	*A. parasiticus*
vacuum	-	-	-	+	*A. parasiticus*

Discussion and conclusions

Our research was methodologically based on previous studies (Chen et al. 2002; Criseo et al., 2001), with the aim at detecting four target genes necessary for aflatoxin production, one regulatory *aflR* (apa-2) and three structural genes *aflD* (nor-1), *aflM* (ver-1) and *aflP* (omt-1). One to all four of these genes were identified in our isolates, demonstrating aflatoxigenic potential of the both species, which supports the findings of Chen et al. (2002) and Criseo et al. (2001). *A. flavus* and *A. parasiticus* showed similar variety of profiles of aflatoxin genes in terms that the both species displayed four different combinations of the genes (Table 3). In the research conducted by Criseo et al. (2008) lack of variable DNA banding patterns with one to four genes (*aflR*, *aflD*, *aflM*, *aflP*) shown to be typical for the non-aflatoxigenic strains of *A. flavus*. Nevertheless, they did not provide clear evidence of its non-aflatoxigenicity, but several reasons could be taken in account such as complete deletion of a gene, part of or the entire biosynthetic cluster, or to the presence of changes at the primer binding sites. All of these could be strong evidence to support fact how complicated is the process of biosynthetic gene amplification for diagnosis of aflatoxin production.

Galo et al. (2012) analysed *A. flavus* strains for the presence of seven aflatoxin biosynthesis genes, targeting the regulatory genes *aflR* and *aflS*, and the structural genes *aflD*, *aflM*, *aflO*, *aflP*, and *aflQ*. The results of the same study showed that three strains, although showing all seven amplification products, were not aflatoxin producers, or they produced aflatoxins in quantities below the minimal detectable level. It is likely that one or more of the other genes involved in aflatoxin biosynthesis are lacking or carry some deletions in these three strains. Also, in our research, we found all 4 target genes in only 2 strains, but we were unable to provide evidence if these strains were aflatoxin producers or not, so our future studies should examine the eventual biosynthesis of aflatoxins.

Neither EU nor national regulations govern the maximum residue limits for aflatoxins in meat products since, as demonstrated in our research, a low percentage

of aflatoxigenic moulds could be found in these type of products. Nevertheless, finding of aflatoxigenic A. flavus and A. parasiticus strains in Bosnian *sudžuk* underscores the need to consider periodical inspection of mould contamination of one of the most consumed traditional dry sausage in Bosnia and Herzegovina as well as a more thorough control of mycotoxigenic potential of isolates originating not only from the contaminated sausages but also from the other foodstuffs that may be contaminated thus presenting a serious health hazard to consumers.

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Utvrđivanje aflatoksinogenih vrsta roda *Aspergillus* u bosanskom sudžuku

Apstrakt

Cilj istraživanje je bio utvrditi prisustvo Aspergillus vrsta u bosanskom sudžuku, najčešće konzumiranoj trajnoj kobasici u Bosni i Hercegovini i procjeniti njihov aflatoksinogeni potencijal. Prikupljeno je 145 uzoraka bosanskog sudžuka u rinfuzi i vakuum pakovanju sa tržišta na području Sarajeva, od toga 105 uzoraka od individualnih zanatskih proizvođača i 40 uzoraka industrijski proizvedenog bosanskog sudžuka. Ukupno su utvrđena 4 izolata A. flavus i 7 izolata A. parasiticus, dok su jedan ili više gena zaduženih za biosintezu aflatoksina (nor-1, ver-1, omt-1 and apa-2) utvrđeni kod 8 izolata. Aflatoksinogeni sojevi nisu utvrđeni u industrijskim uzorcima sudžuka. Ovo je prvo istraživanje prisustva aflatoksinogenih Aspeegillus vrsta u bosanskom sudžuku.

Ključne riječi: A. flavus; A. parasiticus; aflatoksinogenost; bosanski sudžuk; aflatoksinski geni