Natural occurrences of ochratoxicosis in slaughtered pigs from different regions of Serbia

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Abstract

Samples of blood, kidney and liver per animal were randomly selected from slaughtered pigs (n=60) and analyzed for ochratoxin A. Determination of ochratoxin A (OTA) concentration in samples of kidney and liver was performed by HPTLC after immunoaffnity column clean-up, while for plasma samples, spectrofluorometric procedure was used. Of the 60 plasma samples, 60% contained OTA in the range of 2.5-33.3 ng/mL (mean 3.05±5.0 ng/mL), while the incidence of OTA in kidneys and liver were very similar (70% and 65%). The average OTA concentration in liver was 3.2±4.35 ng/g (1.2-19.5 ng/g) and in kidneys was 3.97±4.47 ng/g (1.3-22.0 ng/g). A statistically significant difference ($p<0.01$) was found between region B. Topola and Kovilj for both liver and kidney samples. Also, in kidney originating from region Kovilj and Senta a statistically significant difference ($p<0.01$) was found. Mean distribution followed the pattern:kidney>liver>serum (100>80.8>77%). The results from these survey indicated that there was a strong correlation between the OTA level in serum and liver as well as in the OTA serum in kidney ($r=0.884$ and $r=0.896$, respectively) while the strongest correlation was found between the OTA level in liver and in kidney ($r=0.970$).

Keywords: Ochratoxin A, Tissue, Residue, Swine, Slaughter.

Introduction

Ochratoxin A is a nephrotoxic mycotoxin produced by fungi such as Penicillium verrucosum and Aspergillus ochraceus (1). This toxin has been detected in many countries around the world (2), predominantly found to occur in cereal and cereal products as a result of poor agricultural practice during the drying of produce or poor storage (3). By this route it was recognized that animal feed could be contaminated with ochratoxin A and could subsequently be transferred to animal products for human consumption such as pigs' kidneys and blood sausages (4, 5). In fact, animal-derived products and tissues for human consumption, especially those that include blood and kidney (6), may well present OTA residues even if the animal has been nourished with feeds contaminated with low levels of OTA. It is a potent renal carcinogen in rodents, causes mycotoxicosis in animals, and this mycotoxin plays a special role in the genesis of swine mycotoxic nephropathy, a common disease in Scandinavia (4, 7). Of greatest concern for human health is its implication in an irreversible and fatal kidney disease in certain countries referred to as 'Balkan Endemic Nephropathy' (8). Animal studies have shown the toxin to be a potent nephrotoxin, immunosuppre-ssant and teratogenic (9, 10), and has been classified by the IARC (1993) (11) as Group 2B on account of its carcinogenicity. Surveys of pigs for OTA in blood and/or edible tissues have been carried out in several countries: Denmark (12), Germany (13), Norway (14), and Poland (15). There are no enough data from Serbia.

The purpose of this work was to monitor the presence of OTA in Serbian slaughtered swine, to investigate the regional distribution of OTA and to determine the distribution of OTA in edible tissues.

Materials and methods

Sample collection

During three month period of investigation, slaughtered pigs from each area were randomly sampled in the slaughterhouses line during meat inspection. Samples of kidney, blood serum and liver

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(n=60) from 659 slaughter pigs without any sign of macroscopic changes of the kidneys were randomly collected. About 50 ml blood/pig was sampled when slaughtered pigs were bled by jugular puncture. Blood samples remained at room temperature for 10-12 h to allow clotting to occur, and were then centrifuged at 3000 g for 15min. Serum was decanted and stored at -18 °C prior to analysis. About 70-100 g of liver and one whole kidney were sampled from each pig. The whole sample was homogenized and stored at - 18 °C before analysis. No preservatives were added.

Standard solutions

Stock standard solutions of OTA (approximately 40 mg/ml) for TLC was prepared by dissolving the solid standard (Sigma Chemical Co., St Louis, MO, USA) in toluene-acetic acid (99 : 1 v/v) (16). Standard solution was prepared by diluting appropriate aliquots of standard solution with toluene-acetic acid (99 : 1 v/v). The solutions were stored below -18 °C and protected from light. The concentration was measured according to AOAC International (1998) (17).

Standard solution of ochratoxin A for spectrofluorimetric analysis was prepared from stock solution (10-4 M ochratoxin A) in buffer which was stored frozen and protected from light. The concentration of the stock solution was determined spectrophotometrically at 380 nm, using a value of 5.680 M-1 cm-1 for the extinction coefficient.

Carboxypeptidase's A.: Carboxypeptidase's A was obtained from Sigma Chemical Co.St. Louis, Mo. Carboxypeptidase's A was prepared in 0.04 M tris (hydroxymethyl) aminomethane sulphuric acid buffer, pH 7.5, 1M sodium chloride (100 U/ml).

Extraction and clean-up for ochratoxin analyses:

Serum: The spectrofluorometric procedure for ochratoxin A analysis (Hult et. al. 1980 (18) has been applied. To a 50 ml nylon centrifuge tube, 2.5 ml of plasma, 5.0 ml of chloroform, and 10 ml for plasma of a solution containing 0.05 M HCI and 0.1 M MgCl2 are added. Extraction of ochratoxin A is carried out during 10 min in a tube-turning apparatus rotating 20 turns per min. The mixture is centrifuged for 10 min at 10000 g. From the bottom of the tube, 3.0 ml of the ochratoxin A-containing chloroform is transferred with a syringe into a 15 ml glass centrifuge tube. The chloroform is washed once with 1.5 ml of water on a tube shaker. The phases are separated by centrifugation with a table centrifuge A 3.0 ml portion of 0.04 M tris (hydroxymethyl)

amino methane buffer, adjusted with sulphuric acid to pH 7.5 (20 °C), is added to the chloroform solution, and ochratoxin A is extracted into the buffer by using a tube shaker. The mixture is centrifuged to obtain a clear buffer solution. A 2.0 ml fraction of the buffer extract is transferred to a round borosilicate glass cuvette. A 100-td solution of carboxypeptidase's A is added [100 U/ml in 0.04 M tris (hydroxymethyl) amino methane sulphuric acid buffer, pH 7.5, 1M sodium chloride]. The sample is cooled in an ice bath for 10 min. The fluorescence excitation spectrum is recorded from 320 to 400 nm, at 450 nm emission. The sample is incubated at 37 °C for 2 h and cooled in an ice bath for 10 min. The fluorescence spectrum is recorded again. The loss of fluorescence at 380 nm is proportional to the concentration of ochratoxin A.

Kidney and liver: The detection of OTA in all samples was performed by HPTLC, following the methodology proposed by Jorgensen (2002) (19). A 25-g sample was extracted with 100 ml ethyl acetate and 10 ml 0.5 M phosphoric acid in 2.0 ml sodium chloride by blending in an ultra-turax for 3 min, then a 10 min pause and again for 2 min. The extract was filtered through paper filter and evaporated to dryness. The residue was dissolved in 2 ml methanol, and 30 ml phosphate buffer (pH 7.3) was added. The cleanup of OTA in the samples was carried out using standard, commercially available immunoaffinity columns (Rida Ochratoxin A column, RBiopharm, Darmstadt, Germany). This solution was centrifuged and passed through the immuno-affinity column. The column was washed with 20 ml water, dried by pressing air through the column, and the ochratoxin A was eluted by 5 ml methanol-acetic acid (98 : 2). This solution was evaporated under a nitrogen stream and the residue was redissolved in 200 ml HPTLC mobile phase (toluene-acetic acid (99 : 1 v/v) ready for analysis. Validations of the methods were performed in accordance with the protocol approved by the Association Official of Analytical Chemists (AOAC) (17).

Thin layer chromatography: The purified extracts were analyzed by instrumental high-performance thin-layer chromatography (HPTLC) on the Camag equipment, Switzerland. The 20 cm x10 cm silica gel 60 HPTLC plates (Merck) were used for separation with various mobile phases. One dimensional TLC was sufficient for the separation and quantification of determined mycotoxins. The residues (20 ml) were spotted (LINOMAT IV), in triplicate, on a commercially precoated silica gel 60 glass plate. The glass plates was developed with toluene-ethyl acetate-88% formic acid (6 :3 : 1 v/v/v) and let dry in a fume hood for 5 min. The TLC plates were examined visually under UV light at 365 nm (CAMAG UV Cabinet, 366/254 nm), the fluorescence intensity of standards and samples were compared. The OTA concentration in the samples was calculated by comparing the area of chromatographic peak of the samples with that of the standard calibration curve by densitometry analysis (TLC SCANNER, ?-324 nm, filter 2, photo mode: fluorescence). The

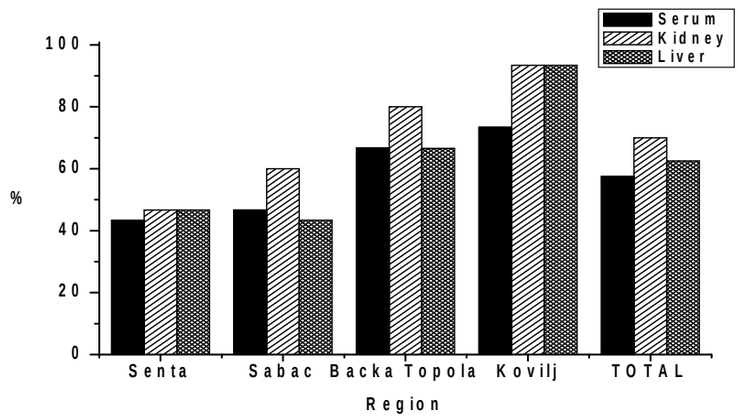

Figure-1. Occurrence of ochratoxin A in slaughtered pigs (n=60)

optimum spectral settings for densitometry were determined in the range 310-340 nm (18). In case the area of the samples was not within in the range of the calibration curve, the sample extract was quantitatively diluted and respotted. The confirmation of OTA was carried out by spraying the plates with sodium bicarbonate according to AOAC International (1998) (17). For every five regions of tissues analyzed, OTA free kidney and liver spiked with OTA at levels of 0.1, 1.0, 2.0 and 35 mg/l, extracted and analyzed sequentially The detection limit for OTA in organs was 0.5 ng/g with an 83% mean recovery. All results are corrected for recovery by use of the actual recovery in the analytical series. For organs the calculated LOD (s/n 3/1) was 0.5 ng/g and LOQ (s/n 7/1) was 1.0 ng/g. The linear equation of the standard calibration curve by densitometric analysis gave R2 = 0.978

Apparatus: A Perkin Elmer LS-5 (USA) spectro-fluorometer with scanning attachment was used. The spectrofluorometer was equipped with a polarizer in the emission pathway, which is necessary when measuring low amounts of ochratoxin. The fluorescence excitation spectrum is recorded from 320 to 400 nm, at 450-nm emission. The detection limit for OTA in plasma was 2 ng/mL with a 78% mean recovery. The linearity of the method from 2 to 200 ng/mL had a correlation coefficient of 0.988. Above 200 ng/mL, the method was not linear, and the recovery of ochratoxin A from plasma decreased to 68%.

Statistical analysis: Differences in the mean levels of OTA contamination across the four 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

The occurrence and distribution of ochratoxin A content into concentration ranges in tissues of slaughtered pigs in the region where samples were collected are summarized in Figure 1 and Table 1.

Serum. The results of this study show that 36 (60%) serum samples, out of 60 were found positive with toxin at levels ranged from 2.5-33.3 ng/mL (mean 3.05±5.0 ng/mL). Of these positive samples, the highest incidence (73.3%), and level were found in the samples originating from region Kovilj (mean 5.31 ng/mL, max 33.3 ng/mL), while the lowest incidence (46.6%) and level of OTA residue were established in the samples originating from region Šabac (mean 1,56 ng/mL, max 5.0 ng/mL). Table 1 presents the results by the number of sample falling into specified concentration ranges and list individual samples exceeding 10 ppb. The majority of contaminated samples (48.3%) contained OTA between 2 and 5 ng/mL, while concentration in five (8.3%) were between 5 and 10 ng/mL. In only two (3.3%) samples OTA concentration was greater then 10 ng/mL (max. 33,3 ng/ml). The results of this study show that the mean level of OTA among the region where samples were collected is very similar, but the incidences of OTA are different (Figure 1).

Kidney. The results of this study reveal that the kidney was the most of the contaminated tissues. OTA was found in 70% of all samples in the range 1.3-22.0 ng/g (3.97±4.47 ng/g). As in serum samples the majority of contaminated samples (40%) contained OTA up to 5 ng/g, while in total 12 (20%) samples exceeded this concentration. In 6 (10%) samples of kidneys, OTA a level was considerably higher and greatly exceed the

Table-1. Distribution of ochratoxin A content into concentration ranges in tissues of slaughtered pigs in the region where samples were collected.

Region	N	Nmber of samples in the range								
		Serum ng/mL								
		<LOD		2a-5		5-10		>10		max.
		n	%	n	%	n	%	n	%	
Backa Topola	15	5	33.3	9	60	1	6.6	-	-	5.20
Kovilj	15	4	26.6	8	53.3	2	13.3	1	6.6	33.3
Šabac	15	8	53.3	7	46.6	-	-	-	-	5.0
Senta	15	7	46.6	5	33.3	2	13.3	1	6.6	18.8
Total	60	24	40	29	48.3	5	8.3	2	3.3	33.3
Region	**N**	**Kidney ng/g**								
		<LOD		0,5a-5		5-10		>10		max.
		n	%	n	%	n	%	n	%	
Backa Topolaa	15	3	20	10	66.6	2	13,3	-	-	8.5
Kovilja,b	15	1	6.6	5	33,3	7	46,6	2	13,3	14.5
Šabac	15	6	40	4	26,6	2	13,3	3	20	22.0
Sentab	15	8	53.3	5	33,3	1	6,6	1	6.6	10.5
Total	60	18	30	24	40	12	20	6	10	22.0
Region	**N**	**Liver ng/g**								
		<LOD		0,5a-5		5-10		>10		max.
		n	%	n	%	n	%	n	%	
Backa Topolaa	15	3	33.3	10	66.6	-	-	-	-	4.1
Kovilja,b	15	1	6.6	10	66.6	3	20	1	6.6	14.5
Šabac	15	7	46.6	5	33.3	-	-	3	20	19.5
Sentab	15	8	53.3	4	26.6	2	13.3	1	6.6	11.5
Total	60	21	35	29	48.3	5	8.3	5	8.3	19.5

N-total number of analyzed samples, LOD- limit of detection (see Materials and methods).
a $p < 0,01$, b $p < 0,01$

permissible levels of this toxins established in Serbia and included those proposed by the European Commission (10 ng/g).

The highest concentrations of OTA were similar in kidney and liver (10% and 8,3% respectively) in contrast to serum (3,3%). With regard to regional distribution of OTA, the occurrence of OTA in the three regions where samples were collected is different and varied between 46.6% (region Senta) to 94% (region Kovilj) (Figure 1). Also, the mean level of contamination was very different and varied between 1.97 ng/g (region Senta) to 6.52 ng/g (region Kovilj).

OTA had almost the same incidence (65%) and mean value in liver as in kidneys. The incidence of OTA was in the range 1.2-19.5 ng/g (mean 3.2±4.35 ng/g). Of these positive samples same incidence was found in the range at 5-10 ng/g and >10 ng/g (8.3%), whereas about 48,3% showed concentration in the range at 0,5-5 ng/g (Table 1). In regard to regional distribution of OTA, the occurrence of OTA among the region where samples were collected is different (46,6% region Senta to 93% region Kovilj) (Figure 1).

The highest mean OTA level (mean 4.53 ng/g) and the highest OTA level (19.5 ng/g) was found in the samples originate from Šabac.

A statistically significant difference ($p<0.01$) was found between region B. Topola and Kovilj for both liver and kidney samples. Also, in kidney originating from region Kovilj and Senta a statistically significant difference ($p<0.01$) was found. The mean distribution followed the pattern kidney>liver>serum (100>80.8>77%). The correlation between the OTA level in serum and in edible tissues is very different. The results from these survey indicated that there was a strong correlation between the OTA level in serum and liver as well as in the OTA serum in kidney (r=0.884 and

r=0.896, respectively) while the strongest correlation was found between the OTA level in liver and in kidney (r=0.970). A similar correlation was found by Curtui et. al. (2001) (20).

Discussion

The results of this study demonstrate the presence of OTA in tissues of Serbian slaughter pigs with an incidence and a mean level of contamination comparable to other European countries (4, 15, 20-22). A comparison with other published data for the occurrence of OTA in tissues of slaughtered swine and contamination level was generally not different from other European countries such as Sweden, Poland, and Germany, in areas of Balkan Peninsula or Canada. The present work indicates that regional differences and seasonal variations were observed. The regional differences and seasonal variations might thus explain the concentration differences in corresponding formulas partly, as could differences in the storage condition of feedstuffs. Since the high OTA concentration is found in blood serum can be suggested that the serum is the most appropriate sample for the control of OTA in slaughtered pigs as well as in pig herds. The fact that ochratoxin A is heterogeneously distributed in a contaminated lot of feed material makes the sampling problematic. It has been shown that an alternative method to monitor the ochratoxin A contamination in the feed is to analyze blood samples from pigs, which reflect the toxin content of the ingested feed. The pig can be looked upon as an in vivo sample collector which forms an average, homogenous blood samples reflecting the ochratoxin A content in its feed. The amount of OTA in the feed given to pigs can be calculated from the values of the OTA concentrations in the blood of pigs. Hult et al. (1980) (18) have reported that the concentration in pig blood plasma (ng/ml) is 1.5-fold higher than in feed (ng/g). Later, other authors have found that the amounts of OTA in pig blood serum were 1.55 and 1.65 respectively, lower than the amounts in feed. Disappearance of OTA from blood was slower than from kidney, liver, and other tissues in pigs.

Serum half-lives after oral administration of OTA was 72–120 h in pigs.

The higher incidence and concentration in kidney than others analyzed tissues is due to the kidney is the main target of OTA, although it has been shown that possible targets of OTA are the liver, the immune system, and brain cells (23-26). This high susceptibility of the kidney is, at least in part, the result of OTA-toxicokinetics. Renal blood flow per tissue weight is extremely high, resulting in the delivery of relative large amounts of OTA as compared to other organs. Furthermore, free OTA is secreted in the proximal tubule and subsequently reabsorbed, mainly in the proximal straight tubule, the thick ascending limb of the loop of Henle and the collecting duct (27, 28). Mechanisms involved in reabsorption are, e.g., H+-dipeptide-cotransporter(s) and nonionic diffusion (28, 29). These toxicokinetic features result in an accumulation of OTA in renal tissue, where the highest concentrations have been detected in the papilla and the inner medulla (28). The inhibition of protein synthesis and the damaged energy production in the mitochondria could be considered as the most important factors for degenerative changes in the epithelial cells of proximal tubules where ochratoxin A was detected. The results of present study show that pork tissues as well pork products are frequently contaminated with OTA. Therefore, pork products, especially those that include blood and kidney, are considered an important source of OTA in humans (6).

Conclusion

Considering the similarities with the occurrence of OTA in edible tissues reported in other countries, it seems that these findings in Serbian slaughtered pigs not represent a particular situation. On the whole, the content of the OTA analyzed was very variable in relation to the region where samples were collected and the different tissues examined. The risk assessment for OTA is based on exposure in adults and the continuous accumulation of OTA in the kidneys leading to tubular dysfunction. Thus, it is important to keep the OTA levels in products aimed for human consumption as low as possible. In order to prevent human exposure to various nephrotoxic mycotoxins, mainly ochratoxin A (OTA), via consuming the meat of animals with nephropathy, the timely diagnosis of disease during the meat inspection at slaughterhouses is very important. However, the actual concentration in pork tissues was generally low compared with other sources and may not constitute a health hazard for consumers because the values are below the recommended daily intake of these toxins (11, 30). Monitoring of OTA in edible tissues is essential in order to prevent excessive build-up of this toxin in the human food chain.

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