PE5.02 Content of ochratoxin A in paired kidney, liver and serum samples from healthy Serbian slaughter pigs 107.00

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***Abstract*— Blood serum, kidney and liver sample per animal were collected from slaughtered pigs (n=90). The samples were analyzed for ochratoxin A (OTA) by HPLC methods. Of the 90 liver samples, 26.6% contained OTA in the range of 0.22-14.5 ng/g. The majority of samples (15.5%) contained OTA between 1-5 ng/g, while ochratoxin A in only two (2.2%) samples of liver was greater than 5 ng/g. The incidence of OTA in serum and kidney were very similar (31%, 33.3%), with a maximum concentration of 220.8 ng/mL, and 52.5 ng/g, respectively. The majority of kidney samples (16.6%) contained OTA at low levels (0.01-1 ng/g). The concentrations in a ten samples (11.1%) ranged between 1-5 ng/g, while ochratoxin A in five (5.5%) samples was greater than 5 ng/g. In 2.2% samples of kidneys, OTA levels was considerably higher and greatly exceed the permissible levels of this toxins established in Serbia and included those proposed (10 ng/g) by the SCF, and JECFA. Analyzing the range of OTA levels in serum fourteen samples (15.5%), levels of ochratoxin A ranged from 0.1 to 1 ng/mL. The concentrations in a further eight samples (8.8%) fell between 1-5 ng/mL while the rest of the samples (6.6%) had concentrations greater than 5 ng/mL. The mean distribution in tissues followed the pattern serum>kidney>liver (100>34>17%). A possible difference in regional distribution of OTA in Serbian is suggested. This study shows the presence of OTA in Serbian slaughtered pigs at levels comparable to those reported in other countries.**

Keywords: ochratoxin A, pig, residue

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I. INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin produced by *Penicillium verrucosum* and by several species of the genera *Aspergillus*. OTA occurs in cereals and cereal products, coffee, beans, pulses, grapes, wine, and dried fruits. As cereals are widely used in animal feed, and because OTA is relatively stable in vivo it can also be found in some animal products, especially in pig kidney and liver. OTA has been shown to be nephrotoxic in monogastric animals, such as pigs and poultry, carcinogenic in kidney, teratogenic of the central nervous system and immunosuppressive in laboratory animals [12, 16, 17]. The presence of OA in pig and poultry feeds also raises some concern for the livestock industry. Among farmed animals, pigs are particularly sensitive to OTA. This mycotoxin play a special role in the genesis of Danish porcine nephropathy, and renal disorders observed in other animals a syndrome characterized by contracted kidneys with tubular degeneration, interstitial fibrosis and hyalinization of glomeruli [6]. It has also been demonstrated that OTA accumulates in blood and edible organs, especially kidneys. Therefore, pork products, especially those that include blood and kidney, are considered an important source of OTA in humans [4]. In humans, intake of high amounts of OTA has been linked to Balkan endemic nephropathy (BEN), a chronic nephropathy described in several rural regions of Balkan Peninsula [13] and associated with an increased incidence of tumors of the upper urinary tract. Recently, OTA has been suggested as playing a role in testicular cancer (15). However, causality has not yet been established. Several international expert groups, such as the Joint FAO/WHO Expert Committee on Food Additives [5] and the former European Commission Scientific Committee on Foods [14], have evaluated Ochratoxin A in food.

The purpose of this work was (1) to give an overview of recent data regarding the occurrence of OTA in Serbian slaughtered swine, (2) to investigate the regional distribution of OTA. We will also discuss possible strategies for control of OTA in tissues of slaughtered swine and other pig products.

II. MATERIALS AND METHODS

Sample collection

During six month period (September 2006/February 2007), samples of blood, kidney and liver from each animal from healthy slaughtered pigs without any sign of macroscopic changes of the kidneys (n=90) originating from three different regions of Serbia where there is a significant swine industry were randomly collected in the slaughterhouse during meat inspection. Serum samples were collected from each studied farm and liver and kidneys of corresponding animals. About 50 mL blood/pig was sampled when slaughtered pigs were bled by jugular puncture. Blood samples remained at room temperature for 24 h to allow clotting to occur, and were then centrifuged at 3,000 × *g* for 20 min. Serum was decanted and stored at -20 °C prior to analysis. About 100 g of liver and whole kidney were sampled from each pig. After cutting pieces of kidney for histological examination, the rest of sample was homogenized and stored at -20 °C before analysis. No preservatives were added.

Reagents

OTA (benzene free) were purchased from Sigma-Aldrich Chemie GmbH. Stock concentrated solution was prepared in toluene-acetic acid (99:1 v/v) at final concentration of 1 mg/mL and stored at -20 °C, protected from light. The OTA working solution was prepared by diluting the stock solution with toluene-acetic-acid (99:1 v/v) to ~10µg/mL. The actual concentration of OTA was calculated using UV spectrophotometer set at 333 nm (ε 5,550). After suitable dilutions in water-methanol-acetic acid (50:49:1 v/v/v), the working solution was used to prepare the external calibration curve. A working standard OTA for HPLC was prepared daily just before starting the injection of a series of samples. Other reagents were HPLC grade. All other chemicals were reagent grade or chemically pure.

Extraction and clean-up for ochratoxin analyses

Extraction and clean-up for ochratoxin analyses from serum (0.8 mL) was extracted according to Curtui and Gareis [1] with 15% trichloroacetic acid (0.2 mL) and dichloromethane (1 mL) by vigorous vortexing for 30 s in a 2 mL safe-lock polypropylene conical-bottom centrifuge tube. The mixture was allowed to stand for 24 h at room temperature, and then centrifuged at 14.000×g for 10 min. The lower dichloromethane phase was carefully withdrawn by a Pasteur pipette and transferred to a 1.5 mL safe-lock polypropylene conical bottom centrifuge tube. The acidic phase and the compact precipitate layer formed between the two phases were re-extracted with dichloromethane (0.5 mL) for 30 s on a vortex mixer and then centrifuged for 5 min at 14,000 × g. The pooled dichloromethane extract was evaporated to dryness at 40 °C under a gentle nitrogen flow. The remaining residue was dissolved in methanol (80 mL) and transferred to a 300 µL HPLC vial. The limit of detection (signal/noise: 3/1) was estimated at 0.1 ng OTA/mL, and recoveries was 86.6% (C.V. 9.6%).

Extraction and clean-up for ochratoxin analyses from kidney and liver were performed by the method of Matrella *et al.*, [10] which briefly includes a double extraction with acidic ethyl acetate. The organic phase was removed and extracted with 0.5M $NaHCO_3$, pH 8.4. The aqueous extract was acidified to pH-2.5 with 7M H_3PO_4 OTA was finally back extracted into ethyl acetate (3 mL). The organic phase was evaporated to dryness under N_2 steam, reconstituted in 150 µL mobile phase and a 20 µL aliquot injected. The detection limit for OTA in organs was 0.14 ng/g with a 71% (C.V. =12%) mean recovery from artificially contaminated samples at 3 ng/g (n = 3).

Chromatographic conditions (HPLC)

An aliquot of 20 µL for serum samples and 50 µL for kidneys and liver samples were injected onto a Waters Symmetry Shield RP (Reversed phase) 18, high pressure liquid chromatography column (length and inner diameter 150×4, 6 mm, particle size 5 µm) on a Waters Alliance HPLC system. The column was eluted with 4% acetic acid and acetonitrile (32:68 v/v) at 25°C and a flow rate of 1 mL/min. Measurements were performed by fluorescence detection at wavelengths of 334 nm (excitation) and 460 nm (emission) gains 10. A volume of 10 µL was injected for the standards and 20 µL for the samples. For more accuracy, 40 µL

was re-injected in the case of the samples with an amount of OTA near the detection limit.

III. RESULTS AND DISCUSSION

The occurrence and mean concentrations of ochratoxin A in swine serum, kidney and liver are summarized in Tables 1 and 2.

Table 1. Inscidence of ochratoxin A in tissues of slaughtered pigs

Region	N	Serum (ng/mL)			
		n (%)	*X* ±Sd	C.V.	Range
Vladimirci	30	5 (16.5)	0.1±90.57	2.96	0.33-2.56
Senta	30	13 (43.3)	2.33±6.91	2.96	0.24-35.7
Bogatić	30	10 (33.3)	8.58±40.25	4.69	0.22-221
Total	90	**28 (31.1)**	**3.70±23.6**	**6.37**	**0.22-221**
		Kidneys (ng/g)			
Vladimirci	30	8 (26.6)	0.42±1.2	2.96	0.18-6.5
Senta	30	11 (36.6)	1.14±3.3	2.89	0.17-17
Bogatić	30	11 (36.6)	2.2±9.54	4.33	0.26-52.5
Total	90	**30 (33.3)**	**1.26±5.85**	**4.64**	**0.17-52.5**
		Liver (ng/g)			
Vladimirci	30	11 (36.6)	0.48±0.75	1.55	0.32-2.2
Senta	30	4 (13.3)	0.84±2.95	3.51	0.56-14.5
Bogatić	30	9 (30)	0.56±1.17	2.09	0.22-5.46
Total	90	**24 (26.6)**	**0.63±1.87**	**2.96**	**0.22-14.5**

Table 2. Distribution of ochratoxin A in tissues of slaughtered pigs in the regions where samples were collected

Region	N	Number of samples in the range			
		Serum			
		< LOD	0.1[b]-1	1-5	>5
Vladimirci	30	25	3	2	0
Senta	30	17	6	4	3
Bogatić	30	20	5	2	3
Total	90	**62**	**14**	**8**	**6**
		Kidneys			
		< LOD	0.14[b]-1	1-5	>5
Vladimirci	30	22	5	2	1
Senta	30	19	5	4	2
Bogatić	30	19	5	4	1
Total	90	**60**	**15**	**10**	**5**
		Liver			
		< LOD	0.14[b]-1	1-5	>5
Vladimirci	30	19	5	6	0
Senta	30	26	1	2	1
Bogatić	30	21	2	6	1
Total	90	**66**	**8**	**14**	**2**

N-total number of analyzed samples, n-number of positive samples, *X* -arithmetic mean (conc. below LOD are regarded as zero), C.V.-coeff. of variation. nd-not detectable, [b] LOD -limit of detection (see Experimental Section).

1. *Ochratoxin A in Serum*

OTA contamination assessment showed that 28 (31%) of the analyzed serum samples (n = 90) were contaminated in a very wide range from 0.22 to 220.8 ng/mL (mean levels 3.70 ± 23.59 ng/mL). The incidences of OTA and mean level of contamination in the three regions where samples were collected are very different (Table 1), varying between 16.6% (mean 0.19 ng/g, Vladimirci) to 43.3% (mean 2.33 ng/g, Senta). The highest OTA level 220.8 ng/mL (mean 8.58 ng/mL), with the highest coefficient of variation (4.69) was found in the samples originate from the Bogatić region. Analyzing the range of OTA levels in fourteen samples (15.5%), levels of ochratoxin A ranged from 0.1 to 1 ng/mL. The concentrations in a further eight samples (8.8%) fell between 1-5 ng/mL while the rest of the samples (6.6%) had concentrations greater than 5 ng/mL. The incidences of higher concentrations of ochratoxin A (>5 ng/mL) in serum was similar to those in kidney (6.6 and 5.5% respectively), while ochratoxin A was greater than 5 ng/g in only two (2.2%) liver samples (Table 2).

2. *Ochratoxin A in Kidney*

In contrast, our results showed that the frequency of contamination of OTA was higher in the kidney than in the serum and liver (Table 1). The incidence of ochratoxin A among the three regions where samples were collected varied between 26.6% (Vladimirci) to 36.6% (Senta and Bogatić) with a mean contamination frequency of 33.3%. In regard to regional distribution of OTA, the average OTA concentration in positive samples varied between 0.42 ng/g (Vladimirci) and 2.2 ng/g (Bogatić) where there is the highest concentration of OTA 52.5 ng/g. The majority of samples (16.6%) contained OTA at low levels (0.01-1 ng/g). The concentrations in a ten samples (11.1%) ranged between 1-5 ng/g, while ochratoxin A in five (5.5%) samples was greater than 5 ng/g (Table 2). In 2.2% samples of kidneys, OTA levels was considerably higher and greatly exceed the permissible levels of this toxins established in Serbia and included those proposed (10 ng/g) by the SCF [14], and JECFA [5].

3. Ochratoxin A in Liver

In the present study, OTA was detected in 24 (26.6%) out of 90 liver samples with a much lower mean value (0.63 ng/g) than in kidney (1.26 ng/g) (Table 1). The majority of samples (15.5%) contained OTA between 1-5 ng/g, while ochratoxin A in only two (2.2%) samples of liver was greater than 5 ng/g (Table 2). In regard to the regional distribution of OTA, the occurrence of OTA among the regions where samples were collected is different and varied between 13.3% (Senta) to 36.6% (Vladimirci), but the mean level of contamination was very similar (0.48-0.84 ng/g). The highest OTA level 14.5 ng/g (mean OTA 0.84 ng/g), with the highest coefficient of variation (3.51) was found in the samples from Senta. Comparison with other published data for the occurrence of OTA and contamination level was generally not different from other European countries such as Sweden, Poland, and Germany, or in areas of Balkan Peninsula and Canada (Table 3).

The present work indicates that regional variations and seasonal differences were observed. Geographical origin and season were recognized as the main factor influencing the OTA content of tissues when samples of the three different regions were compared. During period December-February the mean content of OTA in serum samples in the investigated regions was significantly different ($p<0.05$), while the mean content of OTA in liver samples was highly significantly different ($p<0.01$). The regional differences and seasonal variations might thus partially explain the concentration differences in the corresponding formulas, as could differences in the storage condition of feedstuffs. In addition, fluctuations in mould growth and contamination level of cereals may result in seasonal variations in dietary exposure to OTA. The highest mean level of OTA found in Bogatić region, could signal a possible relationship between this region and the Balkan Endemic Nephropathy. With regards to a consumer safety point of view, the literature contains several studies investigating the ration between the levels of OTA in pig kidneys and pig meat [1, 3, 7, 8, 11]. A contamination level for the entire carcass at 25 ng/g pig kidney should secure that the level in meat do not exceed 10 ng/g, based on the estimation that the OTA level in pig meat is approximately 40% of the level in pig kidney. The ration "*content in meat/content in kidney*" varied between 10 and 90%, and can depend on many factors, e.g. the content of OTA in feed, feeding period, feeding in relation to time of slaughtering. The results from these survey indicated that there was a low correlation between the OTA level in serum and liver as well as in the OTA level in kidney and liver ($r=0.319$ and $r=0.341$, respectively) while the strongest correlation was found between the OTA level in serum and in kidney ($r = 0.973$). A similar correlation was found by [11]. The effects of OTA appeared to be longer-lasting than those of other mycotoxins, and possessed cumulative feature. Comparison the data obtained in this trial with other recently published data for the occurrence of OTA in pig edible tissues shows that the found levels are comparable with levels in other European countries [2, 11, 16]. However, it should be remembered when comparing data that factors such as climate conditions during harvest, practices for grain/feed storage etc have influence on the ochratoxin A level in edible tissues. The data obtained in this trial show it should be raises some concern for the livestock industry**.**

IV. CONCLUSION

The Serbian control system for content of OTA in pig kidney is not yet established, and can be regarded as a lack from a consumer safety point of view. However, the actual concentration in pork is generally very low and hence for the consumer the contribution to the total intake of ochratoxin A from pig products is very small compared with other sources [9]. The values are far below the Acceptable Daily Intake (*ADI*) of these toxins [5, 14], but high amounts of very hazardous and relatively heat stable OTA may enter in the food chain without any control system. With the present safety evaluation of ochratoxin A [5, 14], a residue limit of 10 ng/g in pork would not be considered satisfactory from a consumer safety point of view.

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