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Contamination of wild boars' (*Sus scrofa*) muscles with tetracycline antibiotic from oral-delivered rabies vaccine baits

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ABSTRACT

Tetracycline (TC) is used as a biomarker for rabies vaccine bait intended for foxes. However, there is a high probability of intake of the vaccine by other species living in the forest ecosystem, including wild boars (*Sus scrofa*), and TC residues can occur in the animals' tissues. In this study, muscle samples from 144 animals were tested for the presence of TC, collected after rabies vaccine distribution. For the quantitative analysis of TC and its 4-epi form, a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method was developed. The results of this study show that TC was found in 53 samples, which represents 37% of all tested animals. The concentrations were in the range 5–286 µg/kg. The preliminary results suggest that the risk exists of contamination of muscle tissue of wild boars with TC from oral-delivery rabies vaccine baits containing the antibiotic. Control should be considered of TC residues in wild boar meat for human consumption.

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Introduction

Tetracycline (TC) residues in food products of animal origin intended for human consumption may adversely affect the health of consumers. It may cause allergic reactions, liver damage, renal failure, as well as intestinal microflora disorders. Children can develop permanent brown discolouration of the teeth due to its chelating property and the formation of a TC–calcium orthophosphate complex. Enamel defects and hypoplasia can occur and the growth of teeth and bones can be disturbed (Kline et al. 2012). In addition, residues of antibacterial agents in products consumed by humans may contribute to the rise of drug resistance and the spread on a large scale of resistant bacteria strains (Chopra and Roberts 2001). As a consequence, failure of antibiotic therapy in clinical therapy in humans can occur. It has been proven that even small doses of the antibiotic taken with food for a long time may contribute to the formation of drug-resistant bacterial strains.

Therefore, in order to ensure the proper quality of animal origin products, a systematic control of TC residues with other antibacterial agents in various material of animal origin has been set. The foods subjected to

control are muscles, kidney, liver, milk, eggs and honey. Taking into account the health of consumers, the European Commission, according to Regulation 37/2010, has established maximum residue limits (MRLs) for TC in most edible products (EC 2010). In muscle tissue, the MRL has been fixed at a concentration of 100 µg/kg. This limit is applied to muscles of all food-producing species. Monitoring of TC residues' presence include the muscles derived from cattle, swine, sheep, goats, horses, poultry, fish and farmed wild animals. However, control currently does not include the material intended for human consumption derived from wild boars, while there is a high probability of contamination of wild boar meat with TC, which is present in the vaccine for foxes.

Although the majority of human rabies exposure is due to domestic animals, wild animals constitute a significant reservoir (Vengust et al. 2011). Due to the fact that foxes are one of the most common wildlife species reported with rabies, the oral immunisation of these animals is obligatory and carried out every year. Vaccines bait takes the form of 4-cm diameter capsules containing the liquid vaccine. This is covered by a special

fishmeal polymer, fat bovine paraffin, which is the bait for the vaccine. The polymer is formulated to contain approximately 150 mg TC (tetracycline hydrochloride) as a biomarker of vaccine taken by foxes. The presence of TC in the vaccine bait was the reason for undertaking the presented research.

The reason why TC is widely used as biomarker for monitoring vaccine bait consumption in a variety of carnivores and rodents is its property of complexing with metal ions including calcium ions, so that it accumulates in highest concentrations in the bones and teeth (Cowan et al. 1984; Kuhne et al. 2000; Rosatte and Lawson 2001). Additionally, TC shows fluorescent properties which enables determination of the percentage of the fox population which has taken the vaccine. Monitoring the effects of oral immunisation of foxes against rabies includes determination by fluorescence microscopy of the TC present in the mandible bones of foxes as a marker of vaccine intake. Previously published TC biomarker studies have assumed that TC baits were 100% effective in biomarking animals which had ingested baits (Johnston et al. 2005). Although foxes are the target species of the vaccine, it is not possible to limit its consumption by non-target species which live in the forest. In addition, the strong fishy odour of the vaccine encourages other animals to consume it. In particular, wild boars, which are typically omnivorous animals with a particularly well-developed sense of smell, may possibly consume many doses of the vaccine (Campbell and Long 2009; Campbell et al. 2011). Apart from accumulation of TC in bones, this compound can also penetrate other tissues, like muscles and remain there for some time. Thus the question arises: is there a risk of TC occurrence in venison, which is becoming an increasingly popular meat among consumers?

The purpose of this study was to determine the risk of TC residues' presence in wild boars muscle tissues from rabies vaccine baits, intended for foxes. To the authors' knowledge, there are no available data and research in the literature about the probability of TC transfer from vaccine to wild animal tissues. For the determination of TC, with its 4-epi form, a simple and labour-effective liquid chromatography–tandem mass spectrometry (LC-MS/MS) method was developed.

Materials and methods

Samples collection

Samples were collected from 144 wild boars, weighing 50–70 kg, during routine, individual or group hunting. The diaphragm muscles were analysed. This type of material was the only available matrix in this study. The tested material originated from animals living in the bait zone, from an area with a wild boar population estimated as one animal/4 ha. Samples were collected for 4 weeks after the end of rabies vaccines distribution. Each portion of collected muscle was homogenised using a laboratory grinder and the samples were stored at -20°C before analysis.

Analytical method

Analytical standards and reagents

Tetracycline hydrochloride and 4-epi tetracycline hydrochloride, analytical reference standards and trichloroacetic acid were obtained from Sigma-Aldrich Chemical Company (St. Louis, Missouri, U.S.A.). Acetonitrile (ACN) was from J.T. Baker (the Netherlands) and formic acid from Fluka (U.S.A.). Syringe 0.22 μm Hydrophilic Polyvinylidene Fluoride (PVDF) Membrane Filters were from Restek (College, PA, U.S.A.).

Sample preparation

An aliquot of 2 g muscle tissue was weighted into a polypropylene centrifuge tube and the internal standard (demeclocycline) was added. For the extraction of TC and 4-epi TC, 6 mL of 5% trichloroacetic acid was used. Next, the sample was vortex-mixed, shaken by rotary shaker and centrifuged for 10 min at $9447 \times \text{rcf}$. Then, 1 mL of extract was filtered through a 0.22- μm PVDF filter into an amber vial.

Liquid chromatography–mass spectrometry

For the quantitative analysis of TC and 4-epi TC, a liquid chromatography–tandem mass spectrometry was applied. An Agilent A Series 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) was used, connected with an API 4000 triple quadrupole mass analyser with a TurboIonSpray source (Applied Biosystems, Toronto, ON, Canada). The ESI was operated in the positive ion mode and MS data acquisition was performed in the multiple reaction monitoring (MRM) mode. The precursor $\rightarrow$ product ion pairs for

TC and 4-epi TC were 445 $\rightarrow$ 410 and 427 with declustering potential DP = 55 V, collision energy CE = 27 V for first transition and 25 V for second transition and cell exit potential CXP = 15 V. The precursor $\rightarrow$ product ion for demeclocycline (IS) was 465 $\rightarrow$ 448 with DP = 60 V, CE = 25 V and CXP = 10 V.

The chromatographic separation was performed on a Luna C18 (50 $\times$ 2.0 mm, 3 μ m) analytical column (Phenomenex) coupled with octadecyl guard column (2 $\times$ 4 mm) (Phenomenex). The mobile phase consisted of solvent A – 0.1% formic acid in water and solvent B – 0.1% formic acid in acetonitrile (v/v). Elution was performed in gradient mode starting from 95% of eluent A which decreased to 40% within 5 min. This composition was maintained for 1.5 min, and then increased to 95% of eluent A. The column was operated at 35°C with a flow rate of 0.4 ml/min. The injection volume was set as 20 μ l.

Validation

The validation procedure was performed in terms of linearity concentrations ranging between 5 and 500 μ g/kg (six point calibration level), precision (repeatability and within-laboratory reproducibility) recovery, specificity, detection limit (LOD) and the limit of quantification (LOQ). For the repeatability purpose, six spiked samples at four concentration levels (5, 50, 100, 150 μ g/kg) were analysed. The laboratory reproducibility was evaluated using results of six spiked samples at the same levels as for repeatability that were measured in three different days. The LOD was calculated on the basis of the signal-to-noise ratio (S/N = 3) on the chromatograms of 20 muscle blank samples. The LOQ was calculated as the lowest TC concentration on the matrix-matched calibration curve that could be quantitated with a precision level not exceeding 20% and with accuracy within 20% of nominal.

Results and discussion

For the quantitative analysis of TC and 4-epi TC, the LC–MS/MS method was developed. The optimal isolation of TC and 4-epi TC was achieved with trichloroacetic acid. For cleanup, the PVDF filters were found the most suitable. The presented fast and simple extraction, without SPE using for cleanup purposes, allows analysis of many samples in a very short time.

In chromatography analysis of TC and 4-epi TC, for appropriate separation of TC and their 4-epi form,

different components of mobile phases like formic acid, citric acid, phosphoric acid, acetic acid using Luna C18 analytical column have been tested. A good efficiency in TCs separation without tailing has been achieved with ion-pairing agents or oxalic acid in the mobile phase (Nakazawa et al. 1999; Carrasco-Pancorbo et al. 2008; Shalaby et al. 2011; Bladek et al. 2012). However, mobile phases containing non-volatile compounds cause problems in LC–MS because of clogging at the interface and a build-up of deposits in the ion source (Oka et al. 1998). In the presented study, based on previous experience in TCs' chromatographic analysis, the mobile phase which consisted of a of 0.1% formic acid in water (A): 0.1% formic acid in acetonitrile (B) let to effective separation of TC and 4-epi TC as sharp and symmetrical peaks (Gajda and Posyniak 2015). The MRM chromatograms of blanks spiked with TC and 4-epi TC and tested muscle samples are shown in Figure 1.

The validation results obtained for the presented method were repeatable and reproducible with the repeatability RSDs for TCs and the 4-epiTCs lower than 10% ($\leq$ 6.9% for TC and 8.9% for 4-epi TC) at all fortification levels and within-laboratory reproducibility lower than 15% ($\leq$ 12.2% for TC and 12.9% for 4-epi TC). A good linear response was observed ($r^2 > 0.99$). The specificity of the method was tested by analysis of blank muscle samples and there was no significant chromatographic interference around the retention time of analytes. The recoveries for TC and 4-epi TC were in the range of 91.8–103.6% at all four fortification levels. The procedure was satisfactory sensitive with a detection limit of 2 μ g/kg and a quantification limit of 5 μ g/kg. The validation data for TC and 4-epi TC analysis in muscle samples are presented in Table 1.

During analyses in 15 tested muscles samples, two forms of TC – parent compound, and a stereoisomer of TC, 4-epi TC, were found. The TC fraction was significantly greater than the fraction of 4-epi TC. In literature data, the purity of TC (tetracycline vs. 4-epi TC) in rabies vaccine baits sampled from the end of the supply chain was compared with the purity of starting material (Johnston et al. 2005). Different levels of TC and 4-epi TC were found, depending on the manufacturer. This indicated that the conversion of TC to 4-epi TC was occurring during the manufacturing process of the fish meal polymer baits (Johnston et al. 2005). It appears that exposure to light and heat, as well as environmental chamber exposure can also cause the conversion of TC to 4-epi TC (Johnston et al. 2005).

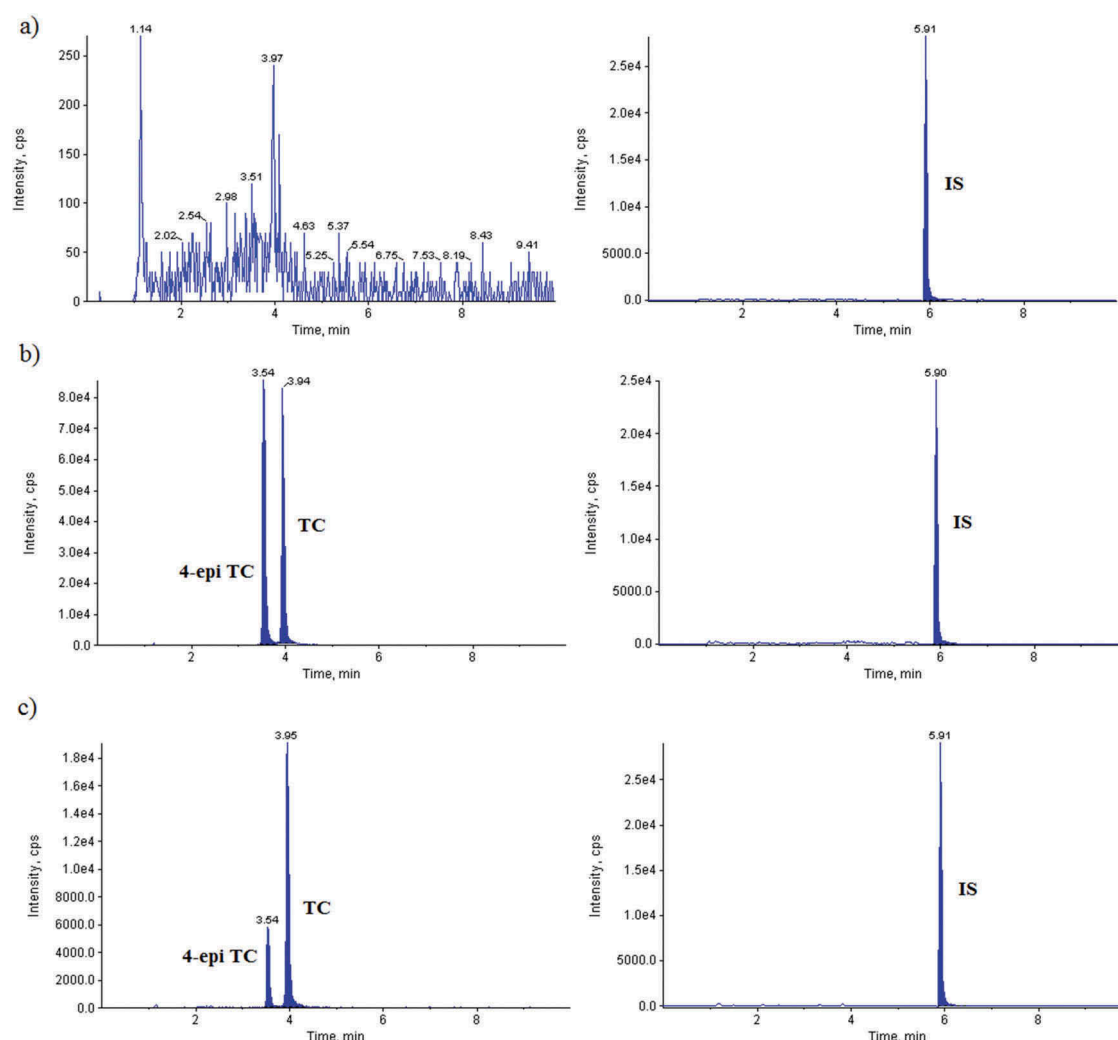


Figure 1. MRM chromatograms of muscle blank sample (a), spiked sample with TC and 4-epi TC at 100 µg/kg for each analyte (b) and tested samples with TC and 4-epi TC at the level of 20 µg/kg and 6 µg/kg, respectively (c).

Table 1. Validation parameters for the method for TC and 4-epi TC analysis in muscle at four concentration levels.

Analyte	Spiked level (µg/kg)	Repeatability (%)	Within-laboratory reproducibility (%)	Accuracy (%)	LOD (µg/kg)	LOQ (µg/kg)
TC	5	6.9	12.2	101.2	2	5
	50	6.8	11.8	96.6		
	100	5.1	9.0	98.1		
	150	5.3	8.8	103.6		
4-epi TC	5	8.9	12.9	93.5	2	5
	50	7.3	12.4	91.8		
	100	5.5	10.9	96.3		
	150	5.2	8.7	97.4		

The presence of TC and 4-epi TC was confirmed in 53 of 144 analysed animals, which represents 37% of samples. The most common detected concentrations of antibiotic found in wild boars muscles were at the range of 5–10 µg/kg (31 samples). In 13 muscle samples, TC was determined at the level of 10–20 µg/kg and 6 samples contained TC at the range of 20–50 µg/kg. There were two samples with TC content above half the MRL

value, with concentrations of 55.0 and 79.9 µg/kg. In one sample, TC was detected at a concentration of 286 µg/kg, much higher than the permitted level. The graphical presentation of concentration ranges detected in samples, as a sum of parent compound TC with the 4-epi TC form, are presented in Figure 2. The exact concentrations of TC and 4-epi TC in wild boar muscles presented for each analyte separately are listed in Table 2.

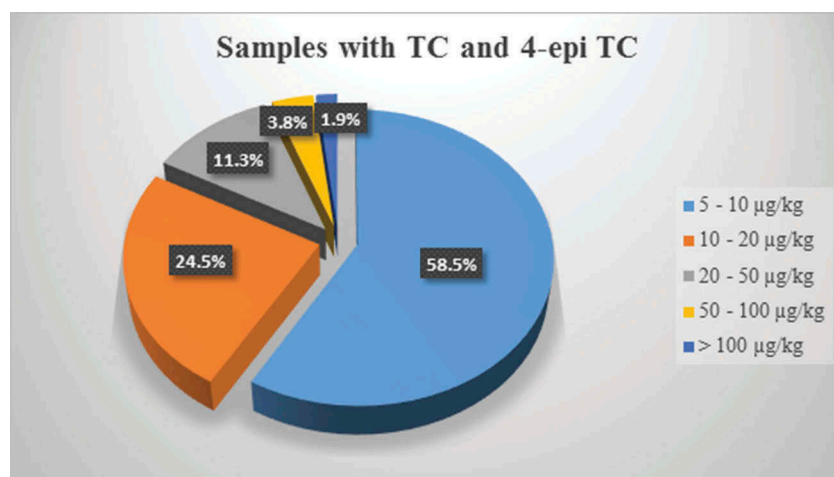


Figure 2. Results of preliminary study for determination of TC and 4-epi TC presence in wild boars muscles from oral-delivered rabies vaccine baits.

Although, most concentrations found in wild boars muscles were below MRL, the frequency of TC detection was relatively high. It should be noticed that even a small amount of antibiotic consumed for a long time is particularly dangerous for consumers, because of antibiotic resistance development. The results of this pilot

study indicate the necessity of further research on the risk of boar meat contamination with TC from rabies vaccine baits. Other kinds of muscles (ham, loin, neck and shoulder muscles) should be analysed, as well as liver and other wild animals' products for human consumption. The number of tested animals from different regions needs to be extended. Different time of

Table 2. The single concentrations values of detected TC and 4-epi TC in wild boars muscles.

[illegible]

vaccine distribution (during and off-season vaccination) should be tested. Evidence of vaccine intake by wild boars was presented by Fletcher et al. (1990), who monitored the number of consumed baits after their distribution in a limited area. It was observed that within 72 h, 88% of 393 baits had gone and at least 52% were taken by wild boars.

Nowadays, a noticeable increase in the consumption of wild boars meat is observed. Boar meat is a highly valued culinary product due to its nutritional properties, characterised by a low fat content of 0.3% to 5%, a high content of whole protein, up to 5%, and taste attributes (Hoffman and Wiklund 2006). The meat has a high quality connected to wild boars living in the free state (Farouk et al. 2007). Probably, the better flavour of wild boar meat derives from the diversity of flora growing in the wild, taken by wild game. Due to increased wild boars populations, the number of wildlife hunts has increased significantly, and this is connected with the greater availability of venison by consumers. Therefore it is particularly important to increase consumers' awareness about the possible danger posed by the presence of TC in products derived from wild boars.

Conclusions

Our preliminary research indicates that wild boar meat may contain traces of TC antibiotic. This study presents, for the first time, the real risk of TC occurrence in tissues of wild animals from vaccines intended for other species. The presented results suggest that control of wild boar meat for TC residues presence should be considered.

Disclosure statement

No potential conflict of interest was reported by the authors.

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