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Monitoring of Stilbenes (GROUP A-1) on Bovine in Kosovo

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Abstract

The Kosovo Food and Veterinary Agency as a Competent Authority for Food Safety, drafts and monitors the National Plan for Residue, in order to obtain an overview of the compliance with the Food and Feed National Law. According to the Directive 96/23 /EC for the Measures on Monitoring of certain substances and their residues in live animals and products of animal origin as well as the National Administrative Instruction 07/2016 regarding prohibition on the use of stockfarming of certain substances having a hormonal or thyreostatic action and of beta-agonist, Competent Authority monitored implementation plan in compliance with EU policies for veterinary drugs residues monitoring. The stilbene substances (Group A1-Stilbenes, stilbene derivatives, their salts and esters) are substances which are strictly banned for use in animals, that are used for food production. Diethylstilbestrol DES is the main representativ active substance of stilbenes, as a syntetic derivate of non steroidal estrogenes. It is possible usage for commercial purposes from farmers, poses a food safety risk. Regarding this publication we are going to present the monitoring of residues in bovines for the last three years. We planned to collect 30 samples, on farm level 15 (urine) respectively in slaughterhouse 15 (meat). Out of total 30 planned samples to be tested, and all of them were actually tested. Samples were tested in Food and Veterinary Laboratory, using ELISA as rapid test. The final result shows that 28 (93.3 %) of them have negative detection and two of them (6.6 %) resulted as suspected in urine matrix on farm level. As a result, we conclude there is a necessity for further testing on confirmatory analysis.

Keywords: Diethylstilbestrol-DES; Elisa; Meat; Residues; Sample; Urine;

Introduction

This publication summarises the monitoring data for the years 2014, 2015 and 2016, to determine the residues presence of unauthorized and banned substances which may pose the a risk factor for public health issues [7]. Kosovo authorities adopted Administrative Instruction 07/2016 which is in compliance with the Directive 96/22/EC concerning the prohibition on the use of Certain Substances in stockfarming having a hormonal or thyrostatic action and of beta-agonists [1]. The following Directive 96/23/EC lays down measures to monitor certain substances and residues thereof, particulary veterinary medicinal products in live animals and animal products. In addition, the Decision 97/747/EC lays down the frequencies of sampling for certain animal products. In particular, Annex I (96/22) covers the prohibited substances called as Group A respectively sub/group A1, belonging to target monitored substances such Stilbenes, Stilbene Derivatives, their Salts and Esters which are of restricted use. Notwithstanding this for therapeutical and zootechnical purposes some substances within Group A excluding stilbenes may be allowed in certain circumstances [4]. Diethylstilbestrol (DES) is the major representative parameter as active substance of Stilbenes, also Hexosterol (HEX) is the minor, but for the practical reasons most of the studies were focused in DES. Stilbenes are efficient anabolic non-steroids, combined with a strong estrogenic activity, also known as Growth Promoter Hormones (GPH). They are synthetics chemical hazardous derivatives, which in 60-70 years they have used [6]. Their use could lead to risk consequences for public health. Apart from carcinogenic, mutagenic, teratogenic, and toxigenic properties, Stilbenes can cause anatomical disorders and physiological disfunctions on hormonal and reproducing systems [8, 9]. According to the Scientific Committee on Risk Analysis, meat originating from animals previously

treated with illegal banned substances for growth promoting purposes “Growth Promotant Hormones” (GPH) results in high risk for consumers. Kosovo Competent Authority for Food and Veterinary applies similar policies to those of European Community regarding illegal and unauthorized chemical residues like. On the contrary, some third countries such as USA, Canada, and Japan, among others, permit the use of GPH in cattle for growth promotion purposes [5]. Naturally and synthetically produced hormones do not differ with regard to their effect mechanism at the receptor, but under certain circumstances synthetic hormones can have more potency and take longer to metabolise and to release from organism [2]. However the aim of this publication is to monitoring the stilbenes, the competent authority planed the National Residue Monitoring Plan (NRMP), it is mean conducting a planned sequence of observations or measurements with the wiev to obtaining an owerviev of the state of compliance whith the food and feed Law. NRMP focuses only in detecting the targeted samples, suspected after having yield non-compliant results. The monitoring data in European Community in terms of Stilbenes-Diethylstilbestrol in bovine analyzed 13.720 samples among which only them, only two samples resulted non-compliant (0.01%) [3].

2. Material and Methods

Sampling

Samples were taken from bovine species in live animals (urine) and primary products of animal origin (tissues like meat and offals). It is important to note that samples were collected at two levels, in farm and at slaughterhouse as targeted reference location. Both levels were located in five different regions in Kosovo. While most of the live and slaughtered animals were of domestic origin, some of them were of imported ones. The samples were collected according to the Standard Operating Procedure Requirements on Sampling according to the NRMP. Every sample had two units which were considered as subsamples. One unit (subsample) of each sample was analyzed as a target and the other parallel unit was treated and conserved for the further necessity analysis as suspected. In order to trace the product, during the sampling process we kept the documentation with relevant and additional information. Samples of plastic bottles for urine matrix and bags for tissue matrix were identified by a serial code, an identifying farmer number in farm and the approval number of slaughterhouses. Samples were placed in cool box and were stored at 4-8 C. The samples were collected in years 2014,2015,2016.

Table 1. Sampling place and number of samples/analyzed substances (parameter)

Sampling region	*Farm No.sample*	*Sl.House No.sample*	***Total***	*Substance Group A1*	***Parameter (active substance)***
01-Prishtina	3	3	6	Stilbenes	Diethystilbestrol
02-Mitrovica	3	3	6	/ / /	/ / /
03-Peja	3	3	6	/ / /	/ / /
04-Prizren	3	3	6	/ / /	/ / /
05-Ferizaj	3	3	6	/ / /	/ / /
	15	**15**	**30**	**30**	**30**

Testing method - ELISA

The method was based on the competitive colorimetric ELISA assay. The drug of interest (Diethylstilbestrol-DES) has been coated in the plate wells. During the analysis sample was added along with the primary antibody specific for the target drug. Afterwards the secondary antibody was tagged with peroxidase enzyme in order to target the primary antibody. ELISA Test Kit had the capacity for 96 wells plate determinations or testing of 42 samples in duplicate including 12 wells for the standards in duplicate as well. Also Kit contents: standards; diethylstilestrol antibody #1; 100xHRP-conjugated antibody #2; diluents; wash solution; stop buffer; TMB substrate; 10xPBS; extraction buffer.

KIT specificity (cross reactivity) was 100 % diethylstilbestrol (DES) parameter as target analytes. Usage materials were Microtiter plate reader 450 nm, incubator, mixer, evaporator, mixer, pipette.

Samples preparation - urine matrix

i.Centrifuged 0.5 mL of the urine sample for 5 minutes., ii. Took out 250 mL of the supernatant, added 50 mL of 10X PBS and 200 mL of methanol. Mixed well., iii. Used 50 mL of the supernatant per well for the assay. Dilution factor was 2.

Samples preparation - meat matrix

i. Preparation of 1xPBS: Mixed 1 vol. of the 10xPBS with 9 vol. of distilled water; ii. Preparation of 1 PBS-Methanol Solution: Mixed 3 vol. of the 1xPBS with 2 vol. of methanol; iii. Preparation of 1xTissue Extraction Buffer: Took all of the powder from the Concentrate of Tissue Extraction Buffer bag put to 200-mL bottle, added 180 mL of distilled water, vortexed it 2 min., left the solution at room temp., for 20 min. Weighted 2 gr homogenized meat sample, added 6 mL of acetonitrile and 2 mL of 1xTissue Extraction Buffer. Vortexed it for 3 min. Centrifuged for 10 min., at room temp., and transferred 3 mL of the supernatant to a new tube. Added 300 mg Tissue and vortexed it for 30 sec., left it at room temp., for 5 min. Centrifuged it for 10 min at room temp. Transferred 2.4 mL of supernatant to a new tube. Used an evaporator to dry the sample. Added 300 uL of 1xPBS/Methanol, vortexed it for 30 sec. Used 50 mL of the sample for the assay.

Testing protocol (flow diagram)

1. Added 50 mL of each Diethylstilbestrol Standards in duplicate into different wells; 2. Added 50 mL of each sample in duplicate into different sample wells; 3. Added 100 mL of antibody #1 and mixed it well by plate manually for 1 min; 4.Incubated the plate for 30 min. at room temp; 5.Washed the plate 3 times with 250 mL of 1x Wash Solution. After the last wash, the plate was dried on paper towels; 6. Added 150 mLof 1x antibody #2 solution. Incubated the plate for 30 min. at room temp; 7. Washed the plate 3 times with 250 of mL 1xWash Solution. After the last wash, the plate was dried on paper towels; 8. Added 100 mL of TMB substrate. Timed the reaction immediately after adding the substrate. Mixed the solution by the plate manually for 1 min. while incubating; 9. After incubating for 15 min. at room temp. added 100 mL of Stop Buffer to stop the enzyme reaction. A standard curve constructed by the mean relative absorbance (%) obtained from each standard against its concentration on logarithmic curve.

DES Standards and concentration level

0.0 ppt (zero);
15 ppt (0.015 µg/kg)
50 ppt (0.050 µg/kg)
150 ppt (0.1 µg/kg)
450 ppt (0.45 µg/kg)
900 ppt (0.9 µg/kg)
2000 ppt (2 µg/kg)

- The Minimum required performance limits (MRPLs) for DES in MUSCUL and Lab. detection capability (CCβpermitted limit) less/equal to respective MRPLs., as followed: CCβ = w(MRPL) +1.64*SD.
- Diethylstilbestrol (DES) MRPLs for meat matrix is 0.3 µg/kg; CCβ = 0.1 µg/kg.
- The Minimum required performance limits (MRPLs) for DES in URINE and Lab. detection capability (CCβpermitted limit) less/equal to respective MRPLs., as followed: CCβ = w(MRPL) +1.64*SD.
- Diethylstilestrol (DES) MRPLs for urine matrix is 1 µg/kg; CCβ = 0.5 µg/kg .

3. Results and Discussion

The detection results were translated by Excel Programme. Two of the urine matrix samples (6.6%) farm level, exceeded the threshold allowance, being 6.655 µg/kg and 8.421 µg/kg, and as such were considered to have reached the suspection limit. Retesting will be performed by confirmative method. The sampling place, animals and farm identity which contain suspected substances are to be disclosed when the documentation and the implementation of the food traceability system in the farm level are being provided. These results will be conserved and presented by the

Competent Authority in order to take the necessary measures in the suspected farms. Suspicious target samples will be dispatched in aim to confirmed using the respective methodology. If these samples are still non-compliant after the confirmatory proof, the study will deepen so as to eliminate the obstacle. The confirmation detects easily the possible origin of hormons, namely to distinguish between the natural and artificial hormons by enabling the identification of their atomic mass.

Table 2. Suspected results for non compliance

Farm Location	*Serial Number*	*Samplimg Reference*	*Substance Parameter Matrice*	***Level of Detection***
F-R.PZ RKS 04	5470/1-3062	PNMM NRMP	STILBENE DES - A1 Urine	**6.655 µg/kg (+ - 1.196)**
F-R.MI RKS 02	5478/1-3069	PNMM NRMP	STILBENE DES - A1 Urine	**8.421 µg/kg (+ - 2.526)**

4. Conlusions

The results of this study pinpoints the necessity to take concrete measures, such as increasing the residue monitoring at all levels of the food chain, starting from the feed up to the slaughterhouse. In addition, previous results of this study indicate that a great deal of attention should be paid to the control of suppliers and warehouses with medical and veterinary equipment, as well as to the production veterinarians, farmers and in general the awareness building among all stakeholders related to the legislation in force. The Competent Authority itself must continue whith the staff training, laboratories should be further enriched whith equipment in order to detect hormonal levels in low limits, and more grounded work needs to be done in the accreditation of laboratories and validation methods. We conlude that the data exchange whith the competent authorities of the countries from which we import meat, live animals, feed, as well as medicinal and veterinary products is of paramount importance. The measures will take in to account all procedures regarding the best practices.

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6. References

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