PharmacologyOnLine

April 30, 2012

Archives • 2012 • vol.1 • 30 - 38

Abuse of pharmaceutical drugs – antibiotics in dairy cattle in Kosovo and detection of their residues in milk

Zana Sulejmani[1]*, Agim Shehi[2], Zehra Hajrulai[3], Elida Mata[2]

[1]University of Prishtina, Faculty of Medicine, Department of Pharmacy - Kosovo;

[2]University of Tirana, Faculty of Medicine, Institute of Public Health - Albania;

[3]University of Skopje, Faculty of Veterinary Medicine, Veterinary Institute - Macedonia.

*z_sulejmani@yahoo.com

Abstract

Introduction: Pharmaceutical drugs – antibiotics are given to livestock by veterinarians for the prevention or/and treatment of disease, but also as a stimulant for animal growth and conversion. Regardless of the way the drugs are inserted into the body of the animal, there will always be residual risk in milk which exceed the maximum level allowed. Thus, given that milk contains high level of drug residue, it can lead to health disorders in humans and in some cases even death.

Aim of study: The main aim of this study is to discover the use of pharmaceutical drugs – antibiotics in dairy cows and to detect betalactam and sulfonamide residues in milk.

Methods and materials used: In 2011, during the period January to August, 80% of dairy farms in the Republic of Kosovo were visited and 127 milk samples were collected from cows treated with pharmaceutical drugs – antibiotics. Samples were analysed ?'3f?'3fin the laboratories of the Veterinary Institute in Skopje using "Elissa" screening method and "HPLC" confirmatory method.

Results: Of 127 samples of milk, 64 of them were contaminated with betalactam residues, while 24 with sulphonamide residues. Of them, the majority, 15 being beta-lactams and 4 sulphonamides. The study shows that over 70% of the total quantity of milk samples analysed contains residues of drugs, while over 20% of them have passed the maximum residue limit allowed.

Conclusion: 70% of the drugs given to dairy cattle in Kosovo are pharmaceutical – antibiotics. Their residues are present in milk at levels high enough to cause severe damage to the entire public health. We conclude that pharmaceutical drugs – antibiotics that are used in humans should not be used in animals too and their provision should be limited to a reasonable and lawful level.

Keywords: pharmaceutical drugs, antibiotics, milk residues

Introduction

Milk is a food substance and nutritious liquid that is essential for normal growth and development of an organism.

Milk offered for sale is a specific food item that requires strict control and enforcement by all legal regulations and relevant state authorities, that must allow its marketing at home and abroad only through special and official permission[1].

Use of antibiotics in dairy farms, and the use of antimicrobial agents at sub-therapeutic levels in dairy animals have always been considered for application[2]. Even Kosovo, as a new and less developed state, is exposed to uncontrolled use of antibiotics in dairy cattle. So even in this country there is a need to monitor the presence of antibiotic residues in food products such as milk.

However, the visible absence of veterinary structures has caused the spread of uncontrolled use of medications to prevent and treat infections in dairy cattle, and, unfortunately, now its use has become so unprofessional that it interferes in the farmer's way of giving antibiotics. This, not only, is damaging to the health and well-being of the animal but it affects greatly the health and wellbeing of human consumers too through consumption of animal products such as milk and meat, thus affecting general public health.

Today, in our globalization age, geography is no longer a factor which can prevent serious disease spreading across borders. Liberalization of market, food systems and free movement of people and animals across borders have greatly reduced geographical barriers[3,4].

Antibiotics are vital drugs in the treatment of infectious diseases in humans and animals. They are used to treat and control diseases caused by bacteria, destroying them or stopping their reproduction, and thus overcoming the infection and allowing the infected body to heal. However, their efficiency can decrease from its broad, continuous, and inadequate use. This can happen not just in medicine but also in agriculture[5].

Beta-lactam antibiotics are most commonly used for the treatment of gram positive and gram negative bacterial infections. They have a broad spectrum of bactericidal action hampering the synthesis of bacterial cell-wall[6].

This group includes all antibiotics, which, in their core ring contain â-lactams, such as: penicillin, b) cephalosporin, c) carbapenems and d) monobactams[7].

Sulphonamides are among the oldest groups of antibiotic/antimicrobial agents. They are widely used in the treatment of bacterial diseases of dairy cattle. They have a wide spectrum of bacteriostatic action, affecting gram positive and gram negative organisms[8,9].

Best-known sulphonamides are: sulfadiazine, sulfamethoxazole, sulphadimidine, sulphadoxine, sulfadimethoxine, sulfamerazine, sulphasalazine, silversulfadiazine and sulfisoxazole, which have the sulfonamide structure as their base[10,11].

Both these types of antibiotics dissolve quickly, easily distribute in all tissues and body fluids, by including the cerebrospinal fluid and fetal circulation. About 90% of these antibiotics binds to plasmatic proteins, and their maximum concentration seen after 3-6 hours. They metabolize in the liver and eliminate by glomerular filtration[12].

The use of antibiotics ignoring any criteria in all cases of disease, be that from cold, viral infection, inflammation or wounding can have a wide negative effect with the drugs left behind in milk and edible tissues. These residues in milk and other food products of animal origin can cause many functional disorders in the human body, such as, for example: a) transfer of bacterial resistance to antibiotics in humans, b) effects of immunopathology, c) carcinogenic effects, d) permanent gene mutation, e) nephropathies or kidney disease, f) liver poisoning, g) reproductive disorders, h) bone marrow poisoning and i) allergic reactions[13].

The aim of the study

The main purpose of this study is to discover the use of pharmaceutical drugs – antibiotics in dairy cattle. Other goal is to detect beta-lactam and sulphonamide residues in the milk manufactured in the Republic of Kosovo.

Material and methods

In order to carry out this research over 80% of dairy farms in the Republic of Kosovo were visited during period January to August of 2011

In this time 127 samples of raw milk were collected, 39 of them during the winter period and 86 of them during the summer.

Samples were taken only from cows treated with pharmaceutical drugs – antibiotics that were last given to them no longer than 2 weeks before the sample collection.

Search and collection of milk samples was conducted in two seasons of the year: during the winter and the summer. This was done to identify the time period during which these drugs are mostly used.

It is observed that the greatest number of cows fell sick during the summer, apparently due to high temperatures that cause proliferation of bacteria and other parasites in water, food, soil and air, in turn, increasing the number of infections and various diseases in dairy cattle.

The assessment was made �'3f�'3fthrough a specific questionnaire, which identified problems in the treatment of cattle with drugs.

Analyses of the samples were done �'3f�'3fin the laboratories of the Veterinary Institute in Skopje using Elisa screening method and HPLC confirmatory method.

To examine the presence of beta-lactam and sulphonamide residues in the samples of raw milk the method used was 'Elisa' for beta-lactams manufactured by Randox-England and for sulphonamides produced by Europroxima in the Netherlands.

Elisa screening method is one of the fastest and most reliable analytical methods for detecting the presence of antibiotics. The testing is easy and shows high sensitivity and fast response.

For the preparation of raw milk samples and in order to detect possible beta-lactams these reagents are used: a) wash buffer (concentrated) of 1 x 32 ml, Randox-England, b) the standards of 6 species, Randox-England, c) conjugate (concentrated) of 3 vials, Randoks-England, d) one shot substrate of 1x15 ml, Randoks-England, e) a bottle of stop solution, Randoks-England and f) re-distilled water.

Whereas, in order to detect sulfonamides these reagents were used: a) ethylacetaat, Merck-Germany, b) iso-octane, Merck-Germany, c) chloroform, Merck-Germany, d) mixture of iso-octane and chloroform in a 2:3 ratio V/V, e) hydrochloric acid 0.1 mol/dm^3, Merck-Germany, f) PBS has been prepared by digesting the mixture of 0.77 g Na_2HPO_4 + 1.88 g KH_2PO_4 + 8.94 g NaCl in one litre of distilled water and adjusted to pH of 7.4±0.2 and g) sulfamerazine standard, Sigma-Aldrich.

In this paper all standards and samples are applied microtitre plate replication. For detection of beta-lactams six standards applied on the microtitre plate have the following concentration: a) 0 ng/ml, b) 0.46 ng/ml, c) 1.0 ng/ml, d) 5.6 ng/ml, d) 10.1 ng/ml and f) 58.9 ng/ml. However, for detection of sulfonamides these standards have concentrations, such as: a) 0 ng/ml, b) 0.125 ng/ml, c) 0.5 ng/ml, d) 0.8 ng/ml, d) 2.5 ng/ml and f) 5.0 ng/ml.

To evaluate the screening method "Elisa" for the analysis of beta-lactams and sulphonamides in milk it is necessary to set some parameters on the basis of set criteria and conforming to the requirements and recommendations by the Rules of the Directive no. 96/23/EC[14]. Parameters to be determined are: a) selectivity, b) precision (accuracy), c) reproducibility, d) limit of detection and e) detection capability (CCâ)[15].

All these parameters are determined by various formulas and their final results are presented in

ISSN: 1827-8620

following table:

Parameters	Total of betalactams	Total of sulphonamides
Calibration curve	Y = -0125 + 0.7744 lnX	Y = -21.758 ln (x) + 40.88
Regression coefficient	0.879	0.9926
Range of concentrations	0.00-58.9 ng / ml	0.125 to 5.0 ng / mL
IC_{50} (concentration at 50 % bounding)	0.65 ng / ml	0.75 ng / mL
Limit of detection (LOD)	1.5 µg / ml	13.3 µg / kg
Detection capability (CCβ)	48.5 µg / kg	54.8 µg / kg
Repeatability	5.05%	7.39%
Reproducibility (precision)	6.28%	8.82%
Recovery (accuracy)	96.18%	97.51%

Results were calculated by Excel program. The absorbance read becomes a percentage (%) of optical density relative to zero standards B_o and it is based on the calibration line assigned to each series of standard solutions and has the following formula:

$$y = a + b * \ln X$$

y: read signal expressed in % of optical density

X: concentration of the substance and

a and b: coefficients

In every batch of samples analyzed for values �'3f�?of R^2 beta-lactams must be at least 0.8278, while the sulphonamides R^2 �'3f 0.98.

Limit of detection is determined by the formula:

$$LOD = X_{average} + 3SD$$

The limit of detection (LOD) determined by Elisa method for beta-lactam residues in milk is 1.5 ìg / ml, while for sulphonamide residues is 13.3 ìg / kg.

Meanwhile, through the confirmatory analysis the presence of antibiotic residues continues to be noted in those samples in which the screening method has shown a detection limit higher than normal.

In our case we used the method of High-Performance Liquid Chromatography with Fluorescence Detection, HPLC/FD in order to detect sulphonamide residues only, since it has not been possible to specifically adjust the HPLC/FD device for beta-lactam detection.

In order to carry out the confirmatory method for sulphonamide residues these reagents were used: a) methanol, Sigma-USA, b) acetonitrile, Sigma-USA, c) water, Sigma-USA, d) acetic acid 98-100%, Merck-Germany, e) hydrochloric acid 36-37%, Merck-Germany, f) formic acid 90%, Merck-Germany, g) ammonia 25%, Merck-Germany, h) acetone, Merck-Germany, i) sodium acetate dissolved 0.01 M with pH = 3.5, j) fluorescamine for synthesis, Sigma-USA, k) sulfadimidine analytical standard, SDM 99.8%, Fluke-Switzerland, l) sulfadiazine analytical standard, 99.0% SDZ, Fluke-Switzerland, m) sulfamethoxazole analytical standard, SMX 99%, Sigma-USA and n) sulfisoxazole analytical standard, SXZ 99.95%, Farmabase-Italy.

Sulphonamide concentrations in the analyzed samples were calculated, through the calibration lines for specific sulphonamides, according to these two formula equations:

$$Y = AX + B$$

Y: signal read by the instrument with the appropriate concentration

X: concentration of the respective substance, A – avoiding the calibration curve

B: circuit that separates the (cuts off) curve from the axis,

and:

$$w = \{(w_s * V) / m\} * 1000$$

ISSN: 1827-8620

w_s: the numerical value of the sulphonamide content in ìg/ml in absorption test

V: the final volume, from which is transferred the test for analysis in ml

m: the numerical value of the mass in g.

Limits of detection and quantification can be determined through the ratio signal/noise, respectively, through these two equations:

$$LOD = 3 * SD \quad \text{and} \quad LOQ = 10 * SD$$

LOD: Limit of Detection

LOQ: Limit of Quantification

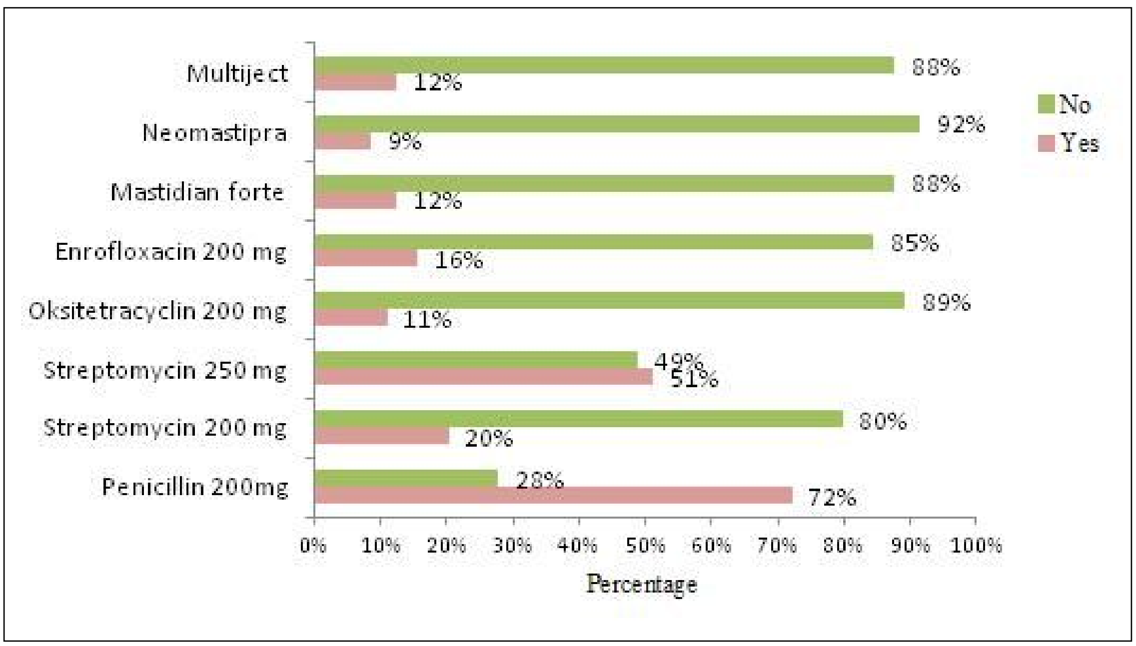
SD: Standard Deviation

The method precision for confirmatory analysis of sulphonamide residues is controlled by the values of repeatability and reproducibility of the method. They are calculated on the level of concentration of 100 µg/kg as is the value of the maximum residue limit (MRL) in conformity with the recommendations of the regulation no. 657/2002[16].

Results

During this research study we have observed that for the treatment of sick cattle in Kosovo beta-lactam and sulphonamide antibiotics are used most commonly. However, not that often, other types of antibiotics are used too such as tetracyclines, aminoglycosides and macrolides.

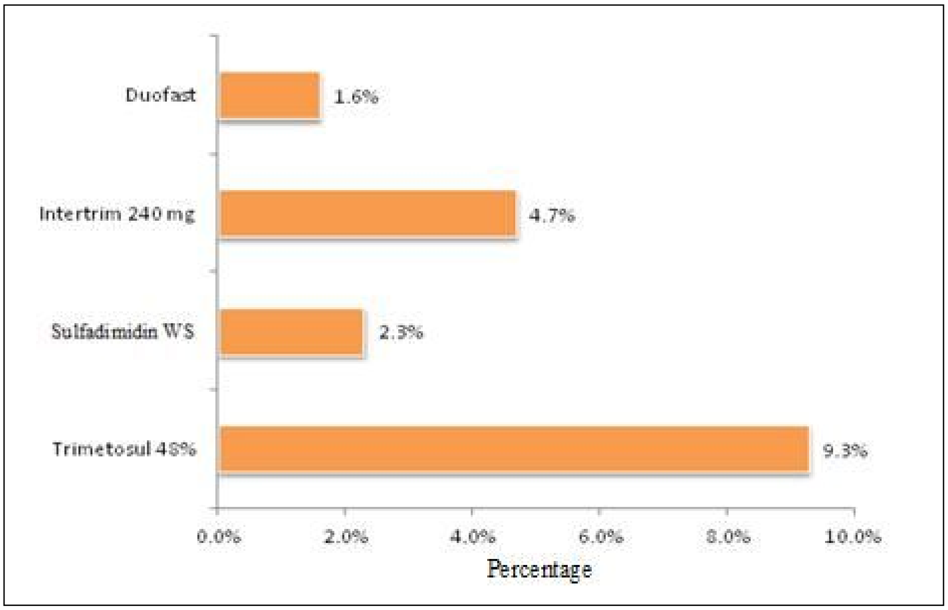
Graph no. 1 presents the percentage of cows treated with beta-lactams[1]:



It can be seen that *penicillin 200mg is one of the beta-lactams mostly used*. 93 cows were treated with pencillin being 72.1% of the total, a statistically significant difference from the number of cows not treated with this antibiotic ($p<0.01$).

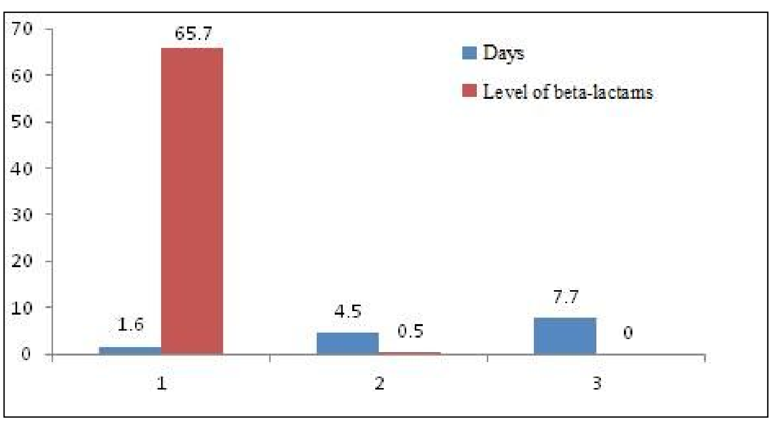
[1]*multiject* = procaine penicillin 100mg + streptomycin 100mg + neomycin 100mg;
[1]*neomastipra* = kloksacilin 200mg + ampicillin 75mg;
[1]*mastidian forte* = colistin sulphate 400.000 IU + metampicillin 200mg;

Graph no. 2 presents the number and percentage of cows treated with sulphonamide[2]:



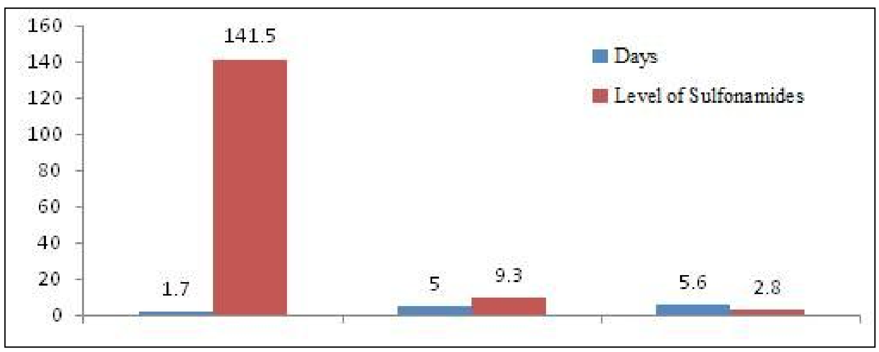
It can be seen that "trimetosul 48%" is mostly used here. 12 cows or 9.3% of the total were treated with this drug, which shows a statistically significant difference when compared to other sulphonamides used ($\chi^2 = 5.10$; $p = 0.01$).

Graph no. 3 present the comparison of beta-lactams residue levels, according to time and delivery of these drugs:



From chart 3 it can be seen that in the first days of their delivery beta-lactam residue level is high and it continues to be so over the following days. By mid-time of their receipt their level significantly lowers, while in last days since their first use the residue level becomes incalculable.

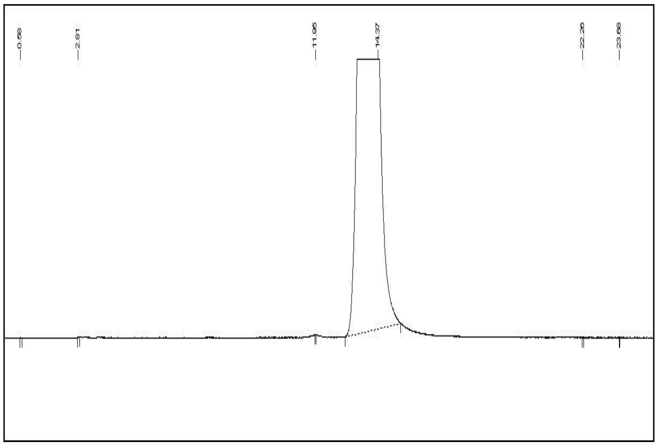
Graph no. 4 present the comparison of sulphonamide residue level according to time and delivery of these drugs



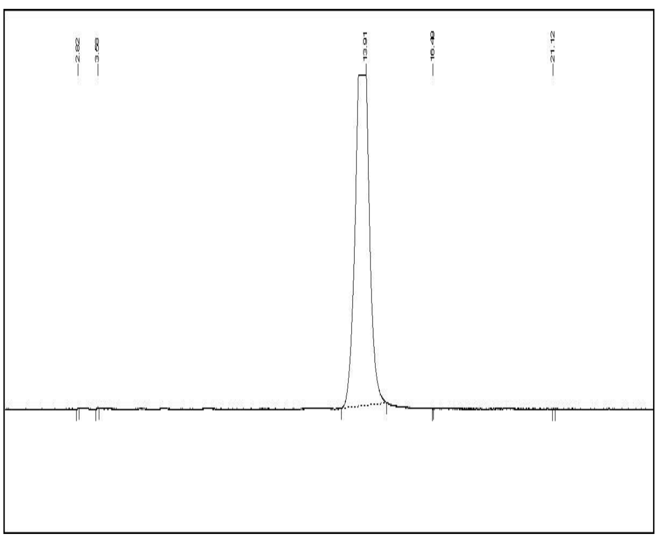
Looking at the chart no. 4 it can be clearly seen that sulphonamide residue level remains high in the first days of their delivery and this high level continues during the following days. By mid-time the drug's residue level significantly lowers, while in last days since their first use their residue level is incalculable.

[1]*duofast* = sulfadiazinum 200mg + trimethoprim 40mg;
[2]*intertrim* = sulfametoksazole 200mg + trimethoprim 40mg;
[2]*sulfadimidin WS* = sulfadimidine 1000mg;
[2]*trimetosul 48%* = sulfadiazine 400mg + trimetoprim 80mg;

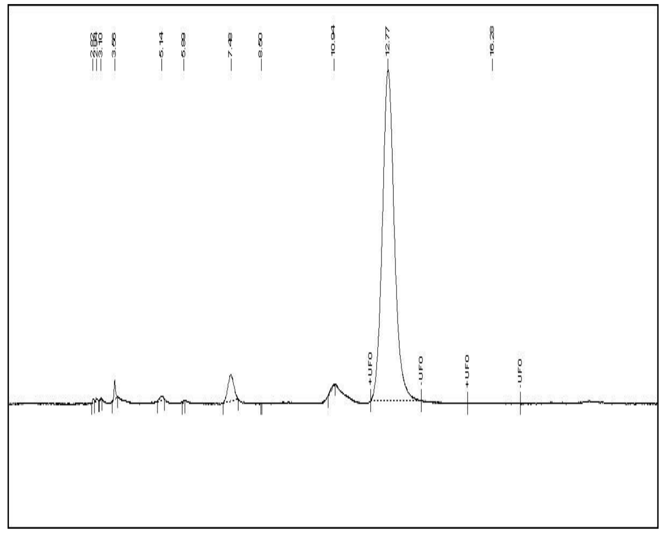
The following tables show chromatograms of sulphonamide residues in 4 samples of raw milk being above the limit of detection (LOD). This limit for the method HPLC/FD is 100 ìg/kg.



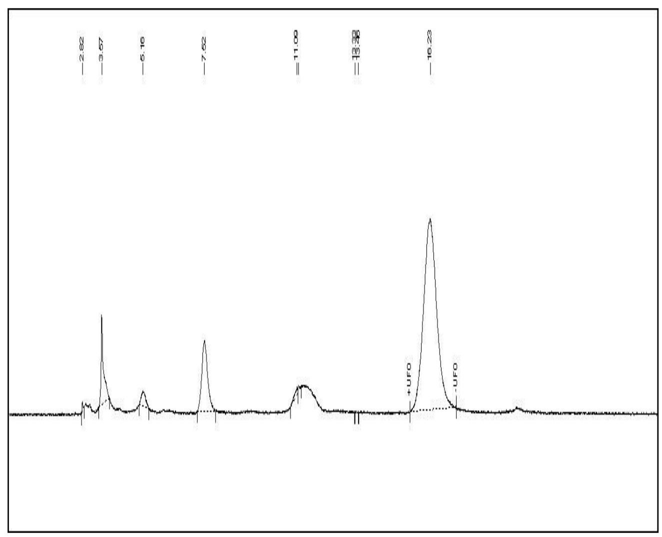
Sample 62: *Sulfadiazina SDZ*



Sample 63: *Sulfadiazina SDZ*



Sample 52: *Sulfadiazina SDZ*



Sample 13: *Sulfadimidina SDM*

During result calculation using the above-mentioned formulas we note that:

a) in the sample no.62 *sulfadiazine SDZ* is detected having a value of *1114.5 μg/kg*, which is passes the limit,

b) in sample no.63 *sulfadiazina SDZ* is also detected with a value of 627 *μg/kg*,

c) in sample no.52 *sulfadiazina SDZ* has a value of *227.6 μg/kg* and

d) in sample no.13 *sulfadimidina SDM* is detected

ISSN: 1827-8620

with a value of *109.9 µg/kg*.

Discussion

Of the whole drugs given to dairy cattle in Kosovo by veterinarians and farmers 70% were pharmaceutical – antibiotics. Their residues were present in milk at high levels, enough to cause severe damage to the entire public health.

Out of 127 samples of milk, 64 were contaminated with beta-lactam residues, and 24 with sulphonamide residues. Wheras 19 samples already passed the limit of detection, the majority 15, being beta-lactam residues, the rest, 4, sulphonamide residues.

In other words, the study confirms that over 70% of the total of milk samples analysed contain drug residues, with 20% of them already passing the maximum residue limit allowed.

According to a survey conducted in Macedonia in 2000 by Velev 79% of drugs - antibiotics used in veterinary medicine are pharmaceutical drugs[17].

Khaskheli et al argue that in Pakistan, during 2006, 36.5% of the total of their raw milk samples were contaminated with beta-lactam residues[18].

Adesiyun and several co-authors have studied the spread of antibiotic residues in raw milk of cattle in Trinidad, in 1997, indicating that 10.8% of all their analyzed samples were contaminated[19].

Other study carried out in Turkey in 2010 by Kaya and Filazi, confirms the presence of beta-lactam residues in 44% of samples among 200 of them analyzed[20]. In their work □'3f□'3fin Iran in 2010 Mohammad Hossein Movasag and several co-authors have shown beta-lactam residues in raw milk to be 5%[21].

Moreover, a study conducted by Shitandi in 2001 in Kenya, confirms that out of the total of 1109 raw milk samples analyzed in Kenia, 21% showed contamination with antibiotic residues[22].

In 1991 in Germany Sanders and several co-authors analyzed around 3000 samples of milk and thereby proved the presence of sulphonamide in 1.1% of samples[23].

Also, in Germany, in 2007, on another survey conducted by Kress and several co-authors during a program for identification of positive samples of milk 1.6% of their cases showed a presence of sulphonamide residues[24].

One other survey made □'3f□'3fin the Netherlands, in 2000, by Abjean applying the examination method in 1100 milk samples of cattle, proves that 9 of the samples or 0.81% of the total were contaminated with sulphonamide residues. However, the survey, failed to show any presence of antibiotic residues when it came to applying the confirmatory HPLC method[25].

A study conducted by Tolentino and several co-authors in 2005 in Mexico using the screening method shows the number of samples detected with sulphonamide residues amounted to 51.3% of the total of analysed samples[26].

Smedley in 1994 when analysing samples using confirmatory method showed that 8 out of the total number were contaminated with sulphonamide residues[27].

Whereas, a study conducted in Turkey in 2007 by Alkan analysed 46 samples using HPLC confirmatory method, and confirmed the presence of sulphonamide residues only in a single sample being above the maximum limit[28].

In a survey by Hyun-Hee Chung in the Republic of Korea in 2008, out of 269 analysed samples using HPLC confirmatory method the presence of sulphonamides was verified only in 4[10].

Conclusion

From the above article we can conclude that pharmaceutical drugs – antibiotics manufactured for human use should not be used in animals too, and the provision of antibiotics should be limited to a reasonable and lawful way

Veterinarians who treat animals in farms are obliged to follow the conditions and regulations for keeping and raising animals, and they should use antibiotics as well as other drugs which are approved for animal use only. Measures must be taken to monitor the presence of drug residues in live animals and animal products, which have pharmacological effects and are harmful to human health.

Veterinarian during his treatment of farm animals, as an evidence of a safe conduct must mark the date, time and mode of application of drugs, the matriculation of the treated animal and the drug warranty. The owner or supervisor of the treated animal must administer veterinary medicine only under veterinary control always following the instructions for use given by the manufacturers and checking the expiry date. In this case the owner of the animal is obliged to keep records of medical prescriptions too.

Acknowledgement

We would like to express our thankfulness to all of those farmers, veterinarians, owners and their families that helped us without laziness in collecting milk samples.

References

1. Donna LS. Pasteurize Why? The dangers Of Consuming Raw Milk. Cornell University, Dairy Science Facts. NY, USA. March 1998.
2. Angulo FJ, Griffin PM. "Changes in antimicrobial Resistance in Salmonella enterica serovar typhimurium." Emerg. Infect. Dis. 2000;(6):436.
3. Boor KJ. Fluid Dairy product quality and safety: looking to the future. J. Dairy Sci. 2001;(84):1-11.
4. Brown P, Will RG, Bradley R, et al. Bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease: background, evolution, and current concerns. Emerg. Infect. Dis. 2001;(7):6-14.
5. Prescott LM, Harley JP, Klein DA. Microbiology. Brown Publishers, 2nd ed; 1993.
6. Droumev DR. Review of antimicrobial agents promoting available. Vet Growth. Res. Commum. 1983;(7):85-99.
7. Ghidini SM, Zanardi E, Varisco G, Chizzolini R. Prevalence of Molecules of ß-lactam Antibiotics in Bovine Milk, in Emily Romagna and Lombardy. ANN. Fac. Medi. Vet. di Parma. Italy, 2002;(22):245-252.
8. Carol A, Gonzalez M, Karyn M. Usher. Determinatio of sulfonamides in Milk Using Solid-Phase Extraction and Liquid Chromatography-Tandem Mass Spectrometry. Department of Chemistry West Chester University, USA. March 11, 2009. 5990-3713EN.
9. Martindale, The Extra Pharmacopoeia, 30th ed.; 208.
10. Chung HH, Lee JB, Chung YH, Lee KG. Analysis of sulfonamide and quinolone antibiotic residues in Korean milk using microbial assays and high performance liquid chromatography. Food Chemistry 2009;(113):297-301.
11. There Drug Bull. Co-trimoxazole use restricted. 33 (12). December 1995; 92-93. doi:10.1136 / dtb.1995.331292. PMIDA 8,777,892 .
12. Richelle RG. Investigation of Safe-Level Testing for Beta-lactam, Sulfonamide, and Tetracycline Residues in Commingled Bovine Milk. Doctoral thesis. Salve Regina University, USA, 5-1-2007.
13. Nisha AR. Antibiotic residues - A Global Health Hazard. Department of pharmacology and Toxicology College of Veterinary and Animal Sciences. Veterinary World, Vol.1 (12):375-377.
14. Council Directive 96/23/EC of 29 April 1996 on Certain Measures to monitor substances and residues thereof in live animals and animal Products Directive 85/358/EEC and repealing Decisions 89/187/EEC and 86/469/EEC and and 91 / 664/EEC. Off. J. Eur. Comm. 1996;(125):10-32.
15. Reybroeck W. Screening for residues of antibiotics and chemotherapeutics in milk and honey. Doctoral thesis. Department of Veterinary Public Health and Food Safety Laboratory of Chemical Analysis, Ghent University;2010.
16. Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical Methods and the interpretation of results OJ L 221; 17.8.2002.
17. Velev R. Palette of registrated drugs and Supporting Medical Supplies for the use in the veterinary medicine in the Republic of Macedonia. Macedonian Veterinary Review 29;(2/3):55-64. Skopje 2000. UDC: 619:615.2.497.7
18. Khaskheli M, Malik RS, Arain MA, et al. Detection of beta-lactam antibiotic residues in market milk. J. Nutrition, 7 (5):682-685. Pakistan 2008.
19. Adesiyun AA, Webb LA. Prevalence of antimicrobial residues in preprocessed and processed Cows milk in Trinidad. J. Food Safety, 1997;(16):301-310.
20. Kaya ES, Filazi A. Determination of antibiotics residues in milk samples. Kafkas Univ. Vet. Factors Derg., 2010;(16):31-35.
21. Movassagh HM, Karami AR. Determination of Antibiotic residues in Bovine Milk in Tabriz, Iran. Global Veterinary Studies 2010;5 (3):195-197. ISSN 1992-6197.
22. Shitandi, A. Detection of antimicrobial drug residues in Kenyan milk. J. Food Safety, 2001;(21):205-214.
23. Sanders P, Guillot P, Dagorn M, Delmas JM. Liquid chromatographic Calf determination of chloramphenicol in tissues: Studies of Stability in muscle. Kidney. and Liver. J. Assoc. Off. Anal. Chem. 1991;(74).
24. Kress C, Seidler C, Hemp B, et al. Experience with an identification and quantification program for inhibitor-positive milk sample. Anal. Chim. Acta 2007;(586).
25. Abjean JP, Delepine B, Hurtaud-Pessel D, et al. Qualitative or quantitative Methods for residue analysis? A Strategy for drug residue monitoring. Proceedings of the Conference EuroResidue IV, 1, 2000. Veldhoven, The Netherlands 8-10 May.
26. Tolentino RG, Perez CA, Gonzales DD, et al. Determination of the presence of 10 antimicrobial residues in mexican pasteurized milk. Intercsiencia, 2005;(3).
27. Smedley, MD. Liquid chromatographic determination of multiple sulfonamide residues in Bovine Milk: Collaborative Study. J. AOAC Int. 1994;(77):1112-1122.
28. Alkan A. The confirmation of used commercial kits in the detection of antibiotics in milk with HPLC (High Pressure Liquid Chromatography). Master thesis. Graduate School of Engineering and Sciences of Izmir Institute of Technology, Izmir, Turkey 2007.