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THREE YEARS OF SURVEILLANCE (2008-10) FOR THE ASSESSMENT OF CONTAMINANTS RESIDUES IN MILK PRODUCED IN KOSOVO

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ABBREVIATIONS AND ACRONYMS

ADI, acceptable daily intake
AFB Aflatoxin B
AFM1 Aflatoxin M1
CVMP, Committee for Veterinary Medicinal Products
ELISA, enzyme-linked immunosorbent assay
EMEA, European Agency for Evaluation of Medicinal Products
EU, European Union
FAO, Food and Agriculture Organization of the United Nations
FDA, Food and Drug Administration
FSCK, Development of a Food safety control System for Kosovo
FVA, Food and Veterinary Agency
GC, gas chromatography
GC-ECD, chromatography with electron capture detection
GC-FPD, Gas Chromatograph/FlamePhotometric Detector
GC-MS liquid chromatography/mass spectrometry
HACCP, Hazard Analysis of Critical Control Points
HPLC, high performance liquid chromatography
JECFA, Joint FAO/WHO Expert Committee on Food Additives
KIA, Kosovo Institute of Agriculture
KFVA, Kosovo Food and Veterinary Agency
KVL, Kosovo Veterinary Laboratory
LC, liquid chromatography
LC-DAD, Liquid chromatography C-diode array detection
LC-MS, liquid chromatography/mass spectrometry
LOD, limit of detection
MAFRD, Ministry of Agriculture, Forestry and Rural Development
MCP, Milk Collection Points
MRL, maximum residue limit
MS, mass spectrometry
NIPH, National Institute of Public Health
NO(A)EL, no observed (adverse) effect level
PISG the Provisional Institutions of self Government
SPUVESEK, Strengthening of Public Veterinary Services in Kosovo
UA, Udhëzimi administrative/administrative order
UHT, ultra-high temperature processing
UNMIK, United Nations Interim Administration Mission in Kosovo
US FDA, United States Food and Drug Administration
WHO, World Health Organization
µg, microgram
€, Euro

ABSTRACT

This paper describes the survey on anti-microbial agents and Mycotoxins in milk as part of a large study to assess public health hazards associated with marketed milk. Samples were collected seasonally during 2008-2010 from individual farms, milk collection points and milk from retail markets (UHT milk). All samples were screened for antimicrobial residues using Delvotest SP (DSM Food Specialties, The Netherlands), Snap tests (IDEXX Laboratories, Westbrook, Maine USA) and Penicillin ELISA test (Immunolab, Germany). The former detects a wide range of anti-microbial, and the latter detects β-lactams and tetracycline's specifically, at levels above maximum residue limits (MRLS) recommended by the European Union (EU).

In this study a total of 1895 milk samples were analyzed by Delvotest SP screening test and all of the positive samples were tested by two screening tests and two confirmatory tests were done to evaluate the contamination of antibiotics during 2008-2010. Positive milk samples yield by Delvotest SP were then tested by Snap beta-lactam test (IDEXX Laboratories, Westbrook, Maine USA) 106 tests, Snap tetracycline test 106 tests, snap sulphamethasine test 106 tests, and Penicillin ELISA test (Immunolab, Germany) 37 tests. The confirmatory analytical methods applied to 80 sample extracts (reacted positive at screening methods).

The qualitative examination of antibiotic residues in 1895 milk samples, during a three year period (2008 to 2010), led to the identification of 131 positive samples (6.91%), 5 ambiguous samples (0.26%) and 1759 negative samples (92.82). In 2008, 25(15.52%) out of 161 samples were positive, in 2009, 52(5.12%) out of 1015 samples were positive and in 2010 54(7.51%) out of 719 samples were positive.

Of all 1895 samples 131(6.91%) were positive for antibiotic residues in cow raw milk in the regions of Kosovo. From 69 UHT milk samples analyzed during 2009 to 2010 two samples were found positive.

The confirmatory analytical methods applied to 80 sample extracts (reacted positive at screening methods) confirmed the presence of tetracyclines (oxytetracycline, tetracycline) by LC-DAD in 5 samples (3 of them with concentration > MRL), the presence of sulfonamides (sulfadiazine, sulfathiazole, sulfamethazine and sulfamethaxazole) by LC-MS in 8 samples (all of them with concentration < MRL) and the presence of beta-lactams (amoxicillin, penicillin G, cefazolin and cloxacillin) by LC-MS in 46 samples (21 of them with concentration > MRL).

The main identified antibiotic families were betalactams (in 90.07% of samples) in average concentrations of 478.22 µg/kg, tetracyclines (in 3.82% of samples) in average concentrations of 14936.66 µg/kg Sulphonamides in (6.11% samples) in average concentration of 7.26 µg/kg.

During 2009 and 2010 the survey on the presence of AFM1 in milk in Kosovo has been performed. In this study 895 milk samples were analyzed by two different screening tests to evaluate the contamination of aflatoxin M1 during 2009-2010 sample collected from individual farms, milk collection points and milk from retail markets. Milk samples (695) in 2009, and (200) in 2010 were tested by ELISA test (Tecna srl, Trieste, Italy), and 9 samples (2009), and 37 samples (2010) were tested with SNAP Afla M1 test (IDEXX Laboratories, Westbrook, Maine USA).

In 2009 from a total of 895 samples examined 20 samples were contaminated with AFM1, positive reaction in tests had 20 samples or (2.88%) out of 695 samples, and 675 (97.12%) were negative, in 2010 from 200 samples in total examined 5 (2.5%) samples reacted positive, and 195 (97.5%) samples reacted negative.
In 2009, the AFM1 concentrations in 695 samples (97.12%) were less than 5 ng/L wail in 20 samples (2.88%) AFM1 was found between 5 and 50 ng/L. In those positive samples AFM1 concentration in milk was in range between 5-10 ng/L (10 samples), 10-25 ng/L (9 samples), and 25-50 ng/L (1 sample). Samples tested with Snap afla M1 test were negative (these samples were randomly selected from positive samples on antibiotics). In 2010, results derived by ELISA test showed that in 195 samples (97.50%) the AFM1 concentrations were less than 5 ng/L and in 5 samples (2.50%) AFM1 was found between 5 and 10 ng/L. All tested samples with SNAP Afla M1 test were negative.
In 2009, the highest concentration of aflatoxin was registered in one October sample which contained 26.59 ng/L. We found the highest number of positive samples in summer (8 samples) and autumn (12 samples). The region where the majority of contaminated samples were found is Peja region while Mitrovica region was without contaminated samples. In 2010, in terms of seasonal distribution, we found the highest number of positive samples in winter (three samples), spring (one sample) and in autumn (one sample). The highest concentration of aflatoxin was registered in one spring sample; it contained 9.81 ng/L, in winter samples ranged between 6.60 and 8.58 ng/L and one sample in autumn contained 6.70 ng/L. The region where all of the contaminated samples were found is Peja region with 100% positives and with no contamination with Aflatoxin M1 in other surveyed regions of Kosovo. Milk samples were taken throughout January to September to study a possible seasonal influence. At the present study, AFM1 levels were detected in UHT milk in both 2009/2010.

KEY WORDS: milk, antibacterial residues, Aflatoxin M1, detection method, public health

RIASSUNTO

Oggetto di questo lavoro di tesi è stata la ricerca di agenti antimicrobici e di micotossine nel latte presente nel mercato in Kosovo allo scopo di valutare i rischi per la salute pubblica associati al consumo del latte stesso.

I campioni di latte sono stati raccolti stagionalmente nel periodo 2008-2010 dalle singole fattorie, dai centri di raccolta del latte e dai supermercati locali (campioni di latte UHT) di diverse regioni del Kosovo.

Tutti i campioni sono stati analizzati, per la ricerca di residui di antimicrobici, con metodi di screening quali: Delvotest SP (DSM Food Specialties, The Netherlands) in grado di rilevare un ampio range di antimicrobici, Snap tests (IDEXX Laboratories, Westbrook, Maine USA) e Penicillin ELISA test (Immunolab, Germany) in grado di rilevare, in modo specifico, la presenza di tetracicline o β-lattamici a livelli al di sopra dei limiti massimi residuali (LMR) raccomandati dall'Unione Europea.

In questo studio, un totale di 1895 campioni di latte raccolti tra il 2008 e il 2010 sono stati analizzati con il Delvotest SP: 106 campioni risultati positivi sono stati sottoposti ad ulteriori test di screening quali SNAP test specifici per le tetracicline, per i β-lattamici (IDEXX Laboratories, Westbrook, Maine USA) e per i sulfamidici e 37 campioni sono stati sottoposti a test ELISA per le Penicilline (Immunolab, Germany) per risalire alla classe di antibiotico presente.

Per la determinazione quantitativa dei residui di antimicrobici, invece, 80 campioni risultati positivi ai metodi di screening sono stati sottoposti ad analisi con metodi di conferma quali LC-DAD per tetracicline e LC-MS per β-lattamici e sulfamidici.

L'analisi qualitativa dei residui di antimicrobici presenti nei campioni raccolti tra il 2008 e il 2010, ha portato all'identificazione di 131 campioni positivi (6.91%), a 5 campioni "dubbi" (0.26%) e a 1759 campioni negativi (92.82%). Nel 2008, 25 campioni dei 161 raccolti (15.52%) sono risultati positivi, nel 2009, 52 campioni dei 1015 (5.12%) sono risultati positivi, mentre nel 2010 i campioni che hanno dato esito positivo sono stati 54 (il 7.51%). Tutti i campioni positivi per la presenza di antibiotici sono risultati essere campioni di latte fresco non pastorizzato mentre, dei 69 campioni di latte di tipo UHT raccolti tra il 2009 e il 2010, solo 2 hanno dato esito positivo.

I metodi analitici applicati a 80 campioni risultati positivi ai metodi di screening, hanno confermato la presenza di tetracicline (ossitetraciclina e tetraciclina) in 5 campioni (3 dei quali con concentrazioni > LMR), la presenza di sulfamidici (sulfadiazina, sulfatiazolo, sulfametazina e sulfametossazolo) in 8 campioni (tutti con concentrazioni al di sotto dell'LMR) e la presenza di β-lattamici in 46 campioni (21 campioni con concentrazioni > LMR).

Nel 2009 e nel 2010 è stata valutata inoltre la presenza di aflatossina M1 in 895 campioni di latte raccolti dalle fattorie, dai centri di raccolta e dai supermercati locali del Kosovo. Sono stati utilizzati due diversi test di screening per valutare la contaminazione di aflatossina M1: tutti i 695 campioni di latte raccolti nel 2009 e i 200 raccolti nel 2010 sono stati analizzati con il test ELISA (Tecna srl, Trieste, Italy) mentre 9 campioni del 2009 e 37 del 2010 (scelti casualmente tra quelli risultati positivi) sono stati testati anche con il test SNAP Afla M1 (IDEXX Laboratories, Westbrook, Maine USA).

Nel 2009, 20 campioni dei 695 totali (2.88%) sono risultati contaminati da aflatossina M1: 10 campioni con concentrazioni tra 5-10 ng/L, 9 campioni con concentrazioni tra 10-25 ng/L e 1 campione con concentrazioni tra 25-50 ng/L. I 675 campioni risultati negativi hanno presentato concentrazioni inferiori ai 5 ng/L.

Nel 2010, i risultati ottenuti con il test ELISA hanno rilevato la presenza di aflatossina M1 in 5 dei 200 campioni analizzati (2.50%), con concentrazioni comprese tra 5 e 10 ng/L.

Tutti i campioni analizzati con il test SNAP Afla M1 sono risultati negativi.

Nel 2009, la concentrazione più alta di aflatossina (26.59 ng/L) è stata registrata in un campione raccolto nel mese di ottobre. Il maggior numero di campioni positivi si è registrato in estate (8 campioni) e in autunno (12 campioni) e la regione con il maggior numero di campioni contaminati è risultata Peja. Mitrovica, invece è la regione in cui non si sono registrate positività.

Nel 2010, in termini di distribuzione stagionale, 3 campioni positivi sono stati raccolti in inverno (concentrazioni tra 6.60 e 8.58 ng/L), 1 campione positivo in primavera (campione che ha presentato anche la concentrazione più alta, 9.81 ng/L) e 1 in autunno (6.70 ng/L). Anche nel 2010, tutti i campioni risultati positivi per l'aflatossina M1, sono stati raccolti nella regione Peja.

PAROLE CHIAVE: latte, antimicrobici, Aflatossina M1, metodi di rilevazione, sanità pubblica

1. INTRODUCTION

To protect the health of the consumer of food of animal origin, one of the most important principles laid down in the EU legislation for the marketing authorization of veterinary medicines, is that food obtained from animals treated with veterinary medicinal products should not contain residues that might constitute a health hazard for the consumer. Antibiotic residues in milk are a rising issue in the recent years in the EU (Stolker & Brinkman, 2005). The health impact of these residues, such as allergies and resistance of micro organisms towards antibiotics, has led to very strict legislation and high penalties for the suspected companies. Food-borne chemical hazards are also a major cause of trade problems internationally (FAO, 2006). In this respect, effective food safety systems support the economic development of countries by providing a sound regulatory foundation for domestic and international trade in food (FAO, 2006).

There is a very limited amount of data available on the evaluation of the situation in regards to monitoring and surveillance programs and actual situation regarding the presence of chemical residues in milk for human consumption and analytical capabilities tests and equipment available for analysis of antibiotics and mycotoxins as presented in EU MRL compliance in Kosovo.

In Kosovo, antibacterial drugs such as beta-lactams, tetracyclines and sulphonamides, are routinely used in veterinary medicine for prevention and therapy of diseases in cattle and in milking cows. No data is available on the presence of penicillin, tetracycline and sulphonamide residues in milk produced and marketed in Kosovo, but for the increasing demand from European countries of food control for veterinary drug residues in milk and meat, this issue is becoming very important also for our consumers. To protect consumer's health and to ensure high quality of food of animal origin, the European Union (EU) regulation 2377/90 (now amended by EU Reg. 470/2009 and 37/2010) set the procedure for establishment of the maximum permitted level of antibiotic residues in milk and meat, since then.

Until 2005, residues of veterinary drugs in Kosovo were governed by the former Yugoslav legislation which only mentioned hormones, sulphonamides and no other veterinary drugs. The MRLs of sulphonamides established by EU, were accepted only for milk, while the residues of other veterinary drugs were not allowed at all. Foodstuffs could only be marketed if they did not contain drug residues at amounts measurable by the officially recognized methods (zero level) (Pravilnik, 1983, 1987). In 2005, the Kosovo legislation concerning the residues of veterinary drugs in foodstuffs of animal origin (UA, 2005), was fully harmonized with the EU and the use of active ingredients, listed in Annex IV of Regulation No. 2377/90 (EC, 1990) was prohibited in farm animals (Administrative instruction, 2005). Thus, to start efficient food residue surveillance and meet international requirements on food safety it is mandatory to adopt improved analytical methods and join testing programs for laboratory proficiency. The major aim of the work was to start a preliminary survey of raw milk, selecting a few representative veterinary drugs among those frequently employed in therapy of milking cows; to facilitate the development of analytical methods for contaminants, strengthening of the expertise and the technical capabilities of the laboratories in conducting

chemical food analysis, principles of method validation, participation in proficiency testing and accreditation for the implementation of legislation. To identify the most efficient analytical test for residue screening and detection to evaluate milk safety to develop monitoring and surveillance program for the detection and identification of antimicrobial residues in milk based on scientific approach, engaging laboratory capabilities, staff, equipment and diagnostic tests and kits available in Kosovo and to assist producers milk facility to establish milk screening tests. To protect the health and rights of consumers since consumers also rely on residue monitoring programs, and to underline the importance of continuous surveillance of antibacterial residues in milk and dairy products.

At the moment the Kosovo Regulation does not report official methods for screening and confirmation, so commercial kits for residue screening were selected on the basis of their accuracy, reproducibility and cost. For the first time all the activities required for a milk surveillance program were performed in Kosovo, including milk sample collection, transport, storage and screening analysis. Post screening analyses were performed by both the researchers from Istituto Zooprofilattico Sperimentale delle Venezie and Department of Public Health, Comparative Pathology and Veterinary Hygiene, Padova University in Italy as a free collaboration. Also for the first time Kosovo has participated in the program for proficiency testing in the Trieste project for Aflatoxin M1 testing.

1.1 STATE OF THE ART: THE SURVEILLANCE OF FOODS OF ANIMAL ORIGIN IN KOSOVO

1.1.1 Background

Kosovo's dairy sector is one of the key sectors in development of agriculture continuing to recover after the war in 1999, when at least half of livestock production was missing. Milk production is widespread throughout Kosovo, as the number of commercial farms, milk collection centers and milk processing facilities are constantly increasing and with that the quantity of milk is increasing rapidly. The Kosovo dairy sector is poised to take a giant step forward with the increase production of high quality milk. This production must be accompanied by the manufacturing and marketing of high quality and safe dairy products. In total there are more than 25 dairy processing companies operating in Kosovo (Petrova, 2006). Located throughout Kosovo, these dairies have an annual production of 381,896 tons from which are 58.563.45 tons are from the imports. Total of 440,563.45 tons are consumed in Kosovo by market value for local produced milk annual € 35.934.158 and from imports € 32,463,988 (MAFRD, 2011). The dairy processors' demands for better quality milk is putting increased pressures on MCPs to supply higher quality milk. Presently in Kosovo there is no MCP operating with Good Milk Handling (GMH) standards. There is also a general lack of GMP (Good Manufacturing Practices) being standardized and followed in the processing plants. This inconsistency throughout the dairy food chain results in products of variable quality and inconsistent taste. In order to implement GMP, HACCP and ISO standards the dairy industry needs to have a sustainable laboratory to monitor and control the quality of dairy products for the needs of dairy industry. The dairy industry is not prepared to analyze residue content of dairy products and they must identify the needs for laboratory testing of milk and milk products based on results of this survey. These surveys can assist dairy industry in quality laboratory testing. (Petrova, 2006).

1.1.2 Control of antimicrobial residues in milk in Kosovo

Under Council Directive 96/23/EC (EC, 1996) every EU Member State must monitor a set proportion of the total annual production of different food commodities of animal origin for residues. The surveillance program focuses on obtaining samples from animals suspected of containing unlawful drug residues in their tissues (Sundlof *et al.*, 2000; Dey *et al.*, 2003).

Based on the European Union Residue Monitoring Plan, Kosovo has adopted this program and it is obliged to monitor food-producing animals and their products for residues of legally and illegally used veterinary drugs and to present a National Residue Monitoring Plan that takes into account the specific situation in its country.

Aspects that must be covered in the National Plan are a description of the authorities and laboratories involved in the implementation and execution of the National Plan, drugs to be analyzed, methods for screening and confirmation, action levels, animal species, and number of samples to be taken in relation to the number of slaughtered animals in the previous years. For bovine milk, the annual number of samples is 1 per 15,000 tons of the annual production of milk, with a minimum of 300 samples per year. There are

laboratories active at dairy centers in Kosovo and Milk Collection Points (MCPs) with limited testing methods, and our survey needs to identify what testing methods the dairy centers are implementing with what available testing equipment and to assist them in establishing a reliable test system for residue contaminants. (SPUVESEK, 2005)

Laboratory facilities in Kosovo are still developing, but due to the crisis in '90 and after the war in 1999, the activities started to develop step by step, but were not concentrated in food quality analysis since other problems were more important. At the moment all testing services in the dairy industry are offered by governmental institutions such as: Kosovo Food and Veterinary Agency (KVFA), National Institute of Public Health (NIPH), Kosovo Institute of Agriculture (KIA) which includes microbiology, and physical-chemistry analyses of dairy products. Currently the NIPH is in charge of microbiology lab testing and KI) is the physical chemistry lab testing that conducting different tests on milk quality and other food quality tests. The Kosovo Veterinary Laboratories (KVL) has units for Serology, Parasitology, Pathohistology, and Bacteriology, however does not carry out any food analysis. That is the reason why all food samples are collected in Kosovo and then sent to the Veterinary Institute in Skopje for residue analysis, bacteriology and physico-chemical analysis. The tests on residues are conducted by Veterinary Institute in Skopje; KVFA is spending more than 50,000 € annually for licensing needs of dairy processing plants and 40,000 € for testing the imported milk and milk products at Veterinary Institute in Skopje, Macedonia. KVFA did not participate in International monitoring programs for contaminants as well as for external proficiency tests (both microbiological and chemical), or did validated methods or official methods of sampling and analysis (SPUVESEK, 2005).

1.1.3 Development of legislation on food safety control

In the ten years between 1989 and 1999 policy formulation, resources and management for the veterinary, sanitary, phytosanitary and laboratory services were provided directly from Belgrade After the conflict in 1999 these resources were lost and following the 1999 conflict in the Balkans, Kosovo was left with only the remnants of a food control service. Since the end of the 1999 conflict, Kosovo has been under the management of the ONU. The war contributed to the deterioration of the Kosovo population's living standards and decreased agricultural activities.

Kosovo also lost management of the control of food safety. With the UN resolution n. 1244 (UNMIK) the Provisional Institutions of self-Government (PISG) was established among other institutions like the Ministry of Agriculture Forestry and Rural Development (MAFRD) and the Ministry of Health. These two institutions that by means different departments and laboratories execute the basic food control activities. Much donor-funded interventions supported and still are supporting the agricultural sector of Kosovo with the aim to satisfy the domestic food demand and among other objectives prepare the sector for agricultural export production at a later stage. To this end Kosovo must introduce many significant changes for the advancement of the country and its population of 2 million people. In this regard high quality standards of the EU have to be respected and most important the national legislative framework in veterinary and food safety must be approximate or harmonized with the EU requirement ("acquis communautaire").

The food safety policy objective for an EU membership candidate country is to develop and implement an effective food safety control system based on strong science and EU legislation enforced by an integrated official control service under the umbrella of a single food safety agency that ensures a high level of public health and consumer confidence in food.

Due mainly to the post war situation Kosovo is currently experiencing significant difficulties in the development of a comprehensive legislative framework to address the needs of food safety. Apart from the ex-Yugoslav laws (Pravilnik, 1987) that form the basis for food control (Law on public health protection, Law on safety of products, Law on sanitary Inspection), with the adoption of United Nations Security Council Resolution No. 1244 (1999), a number of newly promulgated legislative tools have been adopted and more are in the pipeline. Kosovo's food chain safety legislation is currently a mix of new laws and sub-legal enactments that have been made and promulgated since the end of the war in 1999 and the laws of former Yugoslavia still apply where new laws and sub-legal enactments have not been made. The Veterinary Law 2004/21, promulgated by UNMIK Regulation 2004/28, gives responsibility for official controls on products of animal origin, including food of animal origin, to the Kosovo Veterinary and Food Agency, which is an Executive Agency of the Ministry of Agriculture.

A new food safety law, based on EC Regulation 178/2000, was drafted in summer 2005 and sent to the Kosovo Assembly for adoption in November 2005. A country seeking EU membership must conform to the conditions set out in Article 49 and the principles laid down in Article 6(1) of the Treaty on European Union. These include adopting the common rules and standards that make up the "acquis communautaire". When new member states join the EU and therefore enter the EU single market, transitional measures are put in place to allow time to adapt to the EU's food chain safety standards. However, food and feed that does not meet EU standards cannot be traded with other EU countries on the internal market.

In addition to the umbrella legislation that applies to all food and feed, the EU has adopted targeted legislation on specific food chain safety issues and specific foods. These include the use of pesticides, food supplements, colourings, antibiotics and hormones in food production, addition of vitamins, minerals and similar substances to foods, products in contact with food, meat, and dairy products. All of these needs to be transposed into the food chain safety legislation of Kosovo.

1.1.4 Food Safety Policy

Food safety policy development since the end of the war in 1999 has largely been focused on rebuilding the food chain safety control system along the lines of the general policy priorities and objectives of the European Union. Responsibility for food chain safety policy development is shared between the Ministry of Agriculture, Forestry and Rural Development and the Ministry of Health, creating the possibility of development of divergent and inconsistent policy between the two Ministries, particularly in the absence of a co-ordinating mechanism. However, there has been little real opportunity so far to begin development of national food chain safety policy, due to a lack of robust scientific data and the absence of national monitoring and surveillance program to provide the data for risk analysis (FSCK, 2006). The development of national policy to address Kosovo's particular food chain safety hazards and risks requires a more systematic

approach to data collection and analysis. The objective of food safety policy in the European Union is to protect consumer health and interests while guaranteeing the smooth operation of the single market. In order to achieve this objective, the EU ensures that control standards are established and adhered to as regards to food and feed hygiene, animal health and welfare, plant health, and preventing the risk of contamination from external substances. This policy was developed in the early 2000s, in line with the "Farm to Fork' approach, thereby guaranteeing a high level of safety for food and feed marketed within the EU, at all stages of production and distribution.

Food chain safety policy in EU Member States is formulated on the basis of scientific evidence and the three elements of risk analysis namely risk assessment, risk management and risk communication. The aim is to ensure a high level of protection of human life and health, taking into accounts the protection of animal health and welfare, plant health and the environment. This integrated approach is an underlying principle of all EU food chain safety policy. This is necessary in order to enable appropriate actions to be taken to prevent, reduce or eliminate risks and to ensure the high level of health protection determined as appropriate in the EU.

1.1.5 Organization of food safety control system

The organization of the food chain safety control system needs to ensure that scientific assessment of risk can be undertaken in an independent, objective and transparent manner based on the best available science. Food chain safety and the protection of consumer interests are of increasing concern to consumers, non-governmental organizations, professional associations, international trading partners and trade organizations. EC Regulation 178/2002 therefore establishes a framework which enables stakeholders at all stages to be involved in the development of food chain safety policy and legislation, and establishes the mechanisms necessary to increase consumer confidence in the safety of food on the internal market. This needs to be built into Kosovo's national food chain safety control system. Consumer confidence is an essential outcome of successful food chain safety policy and is therefore a primary goal of all EU actions related to food chain safety. Transparency of legislation and effective public consultation are also essential elements in building this greater confidence. Better communication about food chain safety and the evaluation and explanation of potential risks, including full transparency of scientific opinions, are of key importance. The organization of the food chain safety control system in Kosovo is currently shared between three Ministries, five inspection services, and two levels of Government, resulting in a service that is somewhat fragmented and uncoordinated (FSCK, 2006).

Kosovo therefore needs to ensure that it has developed and implemented its own systems for checking that its food chain safety policy and implementation are in line with EU priorities and objectives. It must also ensure that the EU laws it has transposed into national legislation are accurate and kept up to date, and that those laws are being implemented and enforced effectively. The legal basis of food chain safety control is being established, albeit in a rather fragmented manner. This results in a fragmented picture of food chain safety in Kosovo, which will be of concern to the European Commission's Food and Veterinary Office if the situation has not been addressed by the time the Commission comes to assess Kosovo's readiness to join the

European Union. There is also insufficient monitoring of changes in the underlying EU legal framework of food chain safety control, and therefore little consequential revision and updating of Kosovo's food chain safety legislation. Monitoring and evaluation of the effectiveness of implementation and enforcement of controls that are designed to ensure the safety of the food chain is an area in which Kosovo has made some limited progress so far (FSCK, 2006).

1.1.6 Drug and residue surveillance

One way of increasing consumer protection, and ensuring a better sample flow, is to develop control, monitoring and a surveillance program. Introducing these types of programs would be a major step forward for consumer food safety in Kosovo, because they would give the inspections efficient tools for detecting breaches of the food safety regulations (SPUVESEK, 2005).

Kosovo faces many of the same residue food safety problems as other parts of this geographical region (Antibiotics, Mycotoxins, Dioxins, pesticides etc.). In order to identify and control actual or potential food safety problems, a monitoring and surveillance program should be established for microbiological and chemical risks. Apart from direct monitoring and surveillance of food safety problems, a program for monitoring for residues will be essential for the future ability of Kosovo to export its agricultural products. The residue monitoring program although developed was not implemented in the field; consequently no report on this matter is produced. Safety of food presents the main demand posed by the consumers. This is also supported by local media which are heavily involved in presenting food scares and deficiencies of the food control system in Kosovo. This resulted with low consumer confidence on the products in the market, particularly domestic products. Monitoring and surveillance programs for veterinary and food safety in Kosovo has four objectives: To improve food safety, and consumer confidence in Kosovo, with the final objective of achieving veterinary and food safety standards that will allow unimpeded export of agricultural products to the European Union. Effective surveillance of Kosovo's food safety in accordance with internationally recognized standards needs to be conducted to enable objective risk assessment, facilitate policy development, and generally inform decisions about Kosovo's ability to trade products of animal origin, other foodstuffs and live food animals. International trade in these items will be impossible at worst and severely limited at best, if this data is not available and provided to organizations such as OIE and WHO (SPUVESEK, 2005). Present levels of sampling, testing and analysis in Kosovo are considered to be insufficient to give an overview of the residues and contaminants that currently have the potential to affect the safety of food in Kosovo. There appears to be virtually no testing or analysis of samples or specimens for animal health or food safety control purposes in Kosovo. Our research also discovered that some of the samples that are taken by the official food control services in Kosovo are sent to laboratories in Macedonia and other countries outside Kosovo for testing of samples.

Testing and analysis in Kosovo need to increase dramatically to provide laboratories with samples and specimens to test and in turn provide sufficient data for objective risk assessment and food safety policy development.

This requires a step change in the levels of sampling, testing and analytical activity. However, the analytical ability of the laboratories is severely limited, as the laboratory does not perform analysis for residues that may pose a major health risk to the consumers in Kosovo.

This limits the number of options regarding relevant monitoring and surveillance programs, and is a potential hazard to the consumers of Kosovo. None of the laboratories participate in proficiency testing activities. This puts the reliability of test results in doubt. Control bodies among different institutions lead to continuation of the old system of inspection that includes sampling and laboratory analysis without applying science based risk assessments and risk analysis systems (FSCK, 2006)

Kosovo Food and Veterinary Agency is authorized to undertake testing of samples taken under the plan for residues, also it is authorized to develop and realize an intensive program of sampling and testing to determine the prevalence of residues at the earliest opportunity. Laboratories are regarded both as service providers to the official food chain control services and providers of research data to Kosovo Government food safety and animal health policy-makers to inform risk assessment. The residue monitoring program although developed, it was not implemented in the field. Consequently, no report on this matter is produced. (Murati, 2006).

1.1.7 Quality assurance/quality control and laboratory accreditation

Reaching the standards for a quality analysis and quality control system is another major hurdle for laboratories in Kosovo. International norms require that laboratories operate under a set quality system, e.g., ISO 17025, which specifies quality criteria for operations in a laboratory prior to accreditation. The complex requirements often are underestimated. Thus any method proposed to become official must be validated in a collaborative trial study, resulting in defined method performance characteristics, while the framework for the design and conduction of such collaborative trial studies as well as the statistical evaluation are also defined in appropriate protocols (Horwitz, 1995). Any method that has been successfully validated according to these protocols can be recognised as an official method for use in legal cases or for international trade purpose. Especially where legal proof may become necessary, analytical methods must be subject to validation procedures. The participation to proficiency testing schemes allows laboratories to assess their competence and to prove the reliability of their results. The particularity of this proficiency study was to include the different steps in the strategy of control of antibiotic residues in food: screening, eventually post-screening and confirmation of positive results.

Proficiency testing schemes are a special form of inter-laboratory studies aiming at the comparison of a laboratory's performance against that of similar laboratories and at the evaluation of the implementation of analytical procedures by analysts in different or in the same laboratories (Maier *et al.*, 1993).

These schemes are also closely linked and sometimes directly part of the formal accreditation process of analytical laboratories (Esser, 1995; Kohl, 1996). Laboratory accreditation can be defined as a formal recognition by an authoritative body of the technical competence of a laboratory to perform tests or calibrations (ISO/IEC, 1996), and has also become mandatory for official food control laboratories in Europe. Participation in proficiency testing has become systematic for laboratories (especially official

control laboratories) over the past few years. The various proficiency schemes are designed to assess qualitative results as well as quantitative analysis capabilities. Nevertheless, the use of validated methods is an obligation for accredited laboratories.

These tests are valuable tools for assessing the laboratory's analytical performance against a ''best practice'' benchmark. The organizers of the proficiency program prepare only the test materials, making sure that the latter are homogeneous and stable (at least during the time the tests are being performed by the participating laboratories). The results of such tests are returned by the participating laboratories, together with information about the method used, the calibration approach etc., to the proficiency test coordinator. The assessment of the laboratories' performance is then performed by comparing the results with the ''true'' value (e.g. spiked material) or to the combined results of all other laboratories (relative approach). In the case of investigation of qualitative testing capabilities, the number of false positives and false negatives can serve as a basis for performance assessment. Proficiency testing schemes are an integral part of accreditation and ensure that technical competence is also maintained in the accredited laboratory. It has recently been reported that the percentage of unsatisfactory results in an accredited laboratory (13%) is lower than that in a non-accredited one (41%) (Cortez, 1999).

1.2 ANTIMICROBIAL RESIDUES IN MILK: CAUSES AND CONCERNS

Antimicrobials are classified according to their chemical structure. They can be classified as broad or narrow spectrum, depending on the range of bacterial species against which they are active, or as bacteriostatic or bactericidal on the basis of their mechanism of action. Mechanisms of antimicrobials fall into four categories: inhibition of cell wall synthesis, damage to cell membrane function, inhibition of nucleic acid synthesis or function, and inhibition of protein synthesis. The aim of antimicrobial therapy is to rapidly produce and then to maintain an effective concentration of drug at the site of infection for sufficient time to allow hostspecific and nonspecific defenses to eradicate the pathogen (Prescott, 2000a; Prescott & Walker, 2000). The most commonly used antimicrobials in food-producing animals are the ß-lactams, tetracyclines, aminoglycosides, lincosamides, macrolides, pleuromutilins and sulfonamides. Antimicrobials are administered to animals by injections (intravenously, intramuscularly, or subcutaneously), orally in feed or water, topically on the skin and by intramammary and intrauterine infusions (Mitchell *et al.*, 1998).

Theoretically, all of these routes may lead to residues appearing in foods of animal origin such as milk, meat and eggs (Johnston, 1998). The use of antimicrobials for the treatment or prevention of disease in animals closely followed their uses in humans (Gustafson, 1993), and they were first employed in veterinary medicine for the treatment of mastitis in dairy cows (Gildow *et al.*, 1938; Foley *et al.*, 1946; Spencer, 1950). Today antimicrobial drugs are used to control, prevent and treat infection, and to enhance animal growth and feed efficiency (Tollefson & Miller, 2000). Currently, approximately 80% of all food-producing animals receive medication for part or most of their lives (Lee *et al.*, 2001a). The main infectious diseases treated are enteric and pulmonary infections, mastitis and skin and organ abscesses (Teuber, 2001). The most likely cause of violative drug residues is the failure to observe withdrawal times (Paige & Kent, 1987; Van Dresser & Wilke, 1989; Guest & Paige, 1991; Paige, 1994). Improper maintenance of treatment records or failure to

identify treated animals adequately may lead to their omission (Sundlof, 1989). Fecal recycling, where the drug excreted in feces of treated animals contaminates the feed of untreated animals, can be the cause of residues of certain antimicrobial groups (Bevill, 1984; McCaughey *et al.*, 1990). Unlawful drug residues can also occur as a result of improper use of a licensed product or through the illegal use of an unlicensed substance. Extralabel dosages and use of drugs which have not been approved for the species in question may lead to unlawful residues (Papich *et al.*, 1993; Kaneene & Miller, 1997; Higgins *et al.*, 1999). Residues can also occur in calves fed milk and/or colostrum from cows receiving antimicrobials (Guest & Paige, 1991). In most countries ß-lactams are widely applied in mastitis therapy and are consequently the major reason for failures to satisfy at least dairy control requirements for inhibitory substances (Sternesjö & Johnsson, 1998). The disease status of an animal and the way in which drugs are administered influence the potential for residues. Indeed, contamination of feeding stuffs seems to be an important source of unintended application of antimicrobials. In a survey carried out in Northern Ireland, antimicrobials were detected in 44% of feeds declared by the manufacturers to be free of medication (Lynas *et al.*, 1998). Suspected reasons for antibiotic positive samples are shown in Table 1.

Table 1. Sources of antibiotic residues in milk.

Poor records of treatment
Failure to observe recommended label withdrawal time
Prolonged drug clearance
Treated animal identification problems
Contaminated milking equipment
Multiple dosing
Milker or producer mistakes- accidental transfer into bulk tank
Products not used according to label directions
Lack of advice on withdrawal period
Withholding milk from treated quarters only
Early calving or short dry periods
Purchase of treated cows
Use of dry cow therapy to lactating cows

The presence of antimicrobial residues in milk can have several drawbacks: health aspects like possible hypersensitivity reaction by the consumer, contribution to the development of antibiotic resistance and inhibition of dairy starter cultures used in the production of cheese and yogurt.

Hypersensitivity to penicillin is the most common side effect experienced by human patients with an incidence ranging from 0.7% to 10% of the population (Dayan, 1993). And can potentially suffer allergic reactions if they ingest small quantities, such as dermal reactions, asthma, or anaphylactic shock (Wicher, *et al.*, 1969; Lindemayr *et al.*, 1981; Kanny *et al.*, 1994). ß-lactams appear to be responsible for most of the reported human allergic reactions to antimicrobials (WHO, 1991; Riviere, 1995, Sundlof, 1994; Fein *et al.*, 1995). Sulphonamides and tetracyclines may also cause allergic reactions (Paige *et al.*, 1997). Toxic and allergic reactions in humans and animals caused by tetracyclines have only been observed at therapeutic doses (Berends *et al.*, 2001). Unless administered slowly, i.v. injections of a tetracycline is likely to cause an

animal to collapse (Prescott & Baggot, 1993). Although rare, various skin reactions, including rashes and urticaria may follow the use of tetracycline. Angio-edema and anaphylaxis are among the more severe allergic responses. Tetracycline residues are deposited in bones and teeth and hence can slow down the growth of the skeleton and irreversibly discolour the teeth of children.

Tetracycline may produce gastrointestinal irritation to varying degrees in some individuals. Sulphonamides may produce a variety of side effects either of an allergic nature or by direct toxicity. In a small population of humans, sulphonamide therapy has been known to produce idiosyncratic drug reactions (unpredictable rare events dependent upon the individual response to the drug). These reactions may include drug fever and urticaria. These reactions are usually reversible in nature. Aplastic anemia and thrombocytopenia have been reported as being induced by drug therapy with trimethoprim–sulphadiazine.

Acute toxic effects, although rare, are most commonly associated with overdose or too rapid rates of i.v. drug administration. For example, dogs receiving large doses of sulphanilamide (1 g/kg of body weight) have exhibited increased salivation, vomiting, diarrhoea, hyperpnoea, excitement, muscular weakness, ataxia and spastic rigidity of the limbs. In cats given large doses of sulphanilamide, spasticity of the limbs and dyspnoea have been observed. Although relatively safe compounds, disorders of the hemopoietic system have been observed following the use of sulphonamide drugs for the treatment of diseases in animals. Transient agranulocytosis and mild hemolytic anemia and vesicle haemorrhage have been associated with treatment in calves and mink, respectively. Another concern with antimicrobial residues in milk is a possible shift in antimicrobial resistance patterns in human enteric bacteria, in addition to toxic effects, effects on intestinal microbiota and the immune system are important (Gorbach, 1993; Waltner-Toews & McEwen, 1994; Perrin-Guyomard *et al.*, 2001). The microbiota in the human gastrointestinal tract form an extremely complex, ecological community, containing more than 400 bacterial species (Carman *et al.*, 1993). Administration of antimicrobial agents may cause disturbances in this community (Nord & Edlund, 1990). To what extent disturbances in the ecological balance between host and microorganisms occur depends on the spectrum of the antimicrobial agent, the dose, pharmacokinetic and pharmacodynamic properties, and in-vivo inactivation of the agent (Sullivan *et al.*, 2001), another side effect is the potential build-up of antibiotic resistant organisms in humans, since the food chain is the predominant way of reaching humans of antibiotic resistant zoonotic bacteria (Witte, 1998). From the milk processor's perspective, antimicrobials also interfere with the manufacture of dairy products; concentrations of 1 µg/kg delay starter activity for cheese, butter, and yogurt.

These "inhibitors" also decrease the acid and flavor production associated with butter manufacture, reduce the curdling of milk, and cause improper ripening of cheeses (Molina *et al.*, 2003; Payne *et al.*, 2006). In general, concentrations exceeding MRL are needed for total inhibition of mesophilic and thermophilic starter cultures. However, product quality may be already impaired by low antibiotic levels (Mäyrä-Mäkinen, 1995; Suhren, 1996; Grunwald, 2002).

1.2.1 Safety evaluation of antimicrobial drug residues

To assess the safety of ingested antimicrobial residues national and international committees evaluate data on chemical, pharmacological, toxicological and other, antimicrobial properties of the drugs derived from studies of experimental animals and observations in humans (Fink-Gremmels & van Miert, 1994; Woodward, 1998). In the safety evaluation of veterinary drugs tests undertaken to demonstrate the safety of the substance are performed in order to determine a non observed (adverse) effect level (NO(A)EL.

This level is the basis for calculating an acceptable daily intake (ADI). The ADI is an estimate of the residue, expressed on a body weight basis that can be ingested daily over a lifetime without any appreciable health risk (EC, 2001). After an ADI has been determined, maximum residue limits (MRLs) are determined for various food commodities so that overall residue intake remains below the set ADI in a standard food basket. Finally, to insure that drug residues have declined to a safe concentration in various tissues, and a specified period of drug withdrawal is set for any veterinary medicinal product that should be used for the therapy of food producing animals.

Besides FDA, Food and Agriculture Organization (FAO) and World Health Organization (WHO) by means of scientific committees have established MRLs and ADI also of some veterinary residues in milk for consumer protection (Table 2).

Table 2: Residues of some veterinary drugs in cow's milk for human consumption (FAO &WHO)

Pesticides	ADI[a]	Recommended MRLs[b] (mg L^{-1})	References
Antimicrobial agents:			
Ceftiofur	0-50	100	FAO/WHO (1998)
Sulfonamidine	0-50	25	FAO/WHO (1998)
Dihydrostreptomycin (Streptomycin	0-50	200	FAO/WHO (1998)
Chlortetracycline (Oxytetracycline	0-3	100	FAO/WHO (1997)
Gentamycin	0-4	200	FAO/WHO (1995)
Benzyl penicillin	30	4	FAO/WHO (1995)
Insecticides:			
Cypfluthrin	0-20	40	FAO/WHO (1998)
Cypermethrin	0-50	50	FAO/WHO (1997)
Anthelminthic agents:			
Thiabendazole	0-100	100	FAO/WHO (1998)
Albendazole	0-50	100	FAO/WHO (1998)
Production aids:			
Bovine somatotropins	Not specified	Not specified	FAO/WHO (1993)

[a]ADI (Acceptable Daily Intake): ADI for a food is the health endpoint and represents an estimate of the amount of a chemical residue that can be ingested daily over the lifetime of an individual in the general population without appreciable risk.
[b]MRLs: Maximum residue limits

The overall approach to the safety evaluation of residues of veterinary medicinal products within the EU is very similar to that employed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which undertakes the safety evaluation of residues of veterinary medicines on behalf of Codex Alimentarius.

In the EU this work is performed by the Committee for Veterinary Medicinal Products (CVMP) of the European Agency for the Evaluation of Medicinal Products (EMEA), and in the USA by United States Food and Drug Administration (US FDA).

1.2.2 Maximum Residue Limit (MRL)

Maximum residue limit (MRL) of antimicrobial According to the *Codex Alimentarius* (1997), a residue of veterinary medicine is a fraction of the drug, its metabolites, reaction or conversion products and impurities that remain in food originating from animals treated. The maximum residue limit (MRL) is defined as the maximum concentration of drug (expressed in μg/kg or mg/kg) legally permitted or recognized as acceptable in food. The establishment of maximum residue limits (MRLs) for pharmacologically active substances of authorized veterinary medicinal products in foodstuffs of animal origin is governed by Council Regulation (EEC) No 2377/90 and amendments, and amending Directive 2001/82/EC and Regulation (EC) No 726/2004, both of the European Parliament. The classification of the pharmacologically active substances in Commission Regulation (EU) No 37/2010 follows the classification foreseen in Regulation (EC) No 470/2009 that replace the previous Council Regulation (EEC) No 2377/90, now abrogated. The substances are listed in alphabetical order in two separate tables: one for allowed substances, integrating all substances listed in Annexes I, II, and III of Council Regulation (EEC) No 2377/90, and one for prohibited substances, listed in Annex IV to that Regulation. Since the list of allowed substances is a positive list, the administration to food producing animals of veterinary medicinal products containing pharmacologically active substances, which are not listed in the table of allowed substances, is prohibited. Most developed countries prescribe in their laws sanitary regulations for the use of antimicrobials in livestock, setting the maximum residue limits (MRLs) in foods of animal origin. Table 3 shows some of the MRLs established antimicrobials in milk *Codex Alimentarius*, the United States, European Union and Kosovo.

Table 3 Maximum permitted level of antimicrobial residues in milk (mg / kg) established by Codex Alimentarius, the United States (U.S.), European Union (EU) and Kosovo.

ANTIMICROBIAL MRL (μg/kg)	CODEX	USA	EU	KOSOVO
Penicilina G	4	5	4	4
Ampiciline	-	10	4	4
Amoxiciline	-	10	4	4
Ceftiofur	100	100	100	100
Cefapirine	-	20	60	60
Tetracicline	100(a)	300(b)	100	100
Clortetracicline	100(a)	300(b)	100	100
Oxitetracicline	100(a)	300(b)	100	100
Estreptomicine/ Dihidroestreptomicine	200	-	-	-
Estreptomicine	-	-	200	200
Dihidroestreptomicine	-	125	200	200
Gentamicine	200	30	100	100
Neomicine	1500	150	1500	1500

(a) Sum of tetracycline, chlortetracycline and oxytetracycline. (b) Tolerance limit includes both the sum and individual residue of chlortetracycline, oxytetracycline and tetracycline.

1.2.3 Sampling and Analysis

Commission Decision 97/747/EC provides levels and frequencies of sampling in order to monitor some substances and residues thereof for the animal products milk, eggs, honey, rabbits, and game meat. For bovine

milk, the annual number of samples is 1 per 15,000 tons of the annual production of milk, with a minimum of 300 samples. The Commission Decision 98/179/EC lays down detailed rules for official sampling procedures and official treatment of samples until they reach the laboratory responsible for analysis.

Where checks demonstrate the presence of unauthorized substances or products, or when maximum limits have been exceeded, the provisions of Articles 19 to 22 of Regulation (EC) No 882/2004 will apply.

The sampling strategy to be used in the Residue Plan is specified also in Council Directive 96/23/EC (Annex III) and Commission Decisions 97/747/EC and 98/179/EC. In summary, the residue plan is aimed at surveying and revealing the reasons for residue hazards in foods of animal origin. For the Group A substances, the monitoring is aimed at detecting the illegal administration of prohibited substances. For the Group B substances, the monitoring is aimed at controlling the compliance with MRLs (veterinary drugs) and maximum levels (pesticides) and monitoring the concentration of environmental contaminants. In all cases, sampling is targeted (rather than random) taking into account criteria such as sex, age and species of animal, production system in use and all evidence of misuse or abuse of substances. Sampling is required to be unforeseen, unexpected and to be undertaken at no fixed time and on no particular day of the week.

The sampling levels and frequency are specified by Council Directive 96/23/EC for each species of animal (Annex IV). The samples are sent to the testing laboratory designated to undertake official testing for the substances for which the samples were taken. Analyses may be by screening tests - relatively simple, rapid techniques to clear compliant samples and to identify possible non-compliant samples for further testing, or by confirmatory tests - definitive techniques that identify the residue present and usually measure the concentration. Screening tests include immunoassays (Group A substances), inhibitory substance testing (Group B1 substances) and chromatographic techniques such as thin - layer chromatography and high performance liquid chromatography (HPLC). In the case of Group A substances, the confirmatory tests are based on mass spectrometry, GC-MS or LC-MS/MS. In the case of Group B substances, the confirmatory tests are chromatographic techniques including HPLC, GC-ECD, GC-FPD, GC-MS and LC-MS/MS, except for group B3(c) for which testing is performed by atomic absorption spectrophotometry. For many Group B2 and B3 substances, the same techniques are used for screening and confirmatory testing. Groups of residues or substances to be checked for in milk are shown in table 4.

The approved laboratories apply quality control program to their analytical testing to ensure the accuracy of the results obtained and use validated methods according to EC guidelines, and participate in proficiency schemes and inter-laboratory studies. The approved laboratories are expected to have their tests accredited to the ISO 17025 standard. Quality criteria for residue analysis are described in Commission Decision 93/257/EEC, laying down the reference methods and the list of national reference laboratories for detecting residues, as last amended by Commission Decision 2006/130/EC.

Table 4. Annex II of Council Directive 96/23/EC: groups of residuesor substances to be checked for in milk.

Group name	milk
Group A. Substances having anabolic effect and unauthorized substances	
A6 prohibited substances	x
Group B. Veterinary drugs and contaminants	
B1 antibacterial substances, including sulfonamides & quinolones	x
B2a other veterinary drugs - anthelmintics	x
B2c other veterinary drugs - carbamates and pyrethroids	x
B2e other veterinary drugs – non-steroidal anti-inflammatory drugs	x
B3a other substances and environmental contaminants – organochlorine compounds including PCBs	x
B3b other substances and environmental contaminants – organophosphorus compounds	x
B3c other substances and environmental contaminants – chemical elements	x
B3d other substances and environmental contaminants – mycotoxins	x

Notes: x, determination is mandatory.

1.3 MYCOTOXIN RESIDUES IN MILK: CAUSES AND CONCERNS

The aflatoxins were first discovered in 1959/1960 because of their acute toxicity being responsible for the deaths of many turkey poultry in East Anglia; whereas and young game birds are amongst the animals most sensitive to this acute form of poisoning (Blount, 1960).

Mycotoxins are a group of toxic chemical compounds produced by certain strains of fungal species when they grow under favourable conditions on a wide variety of different substrates. Aflatoxin M1 and M2 are oxidative metabolic products of aflatoxin B1 and B2, respectively, and are found in milk obtained from livestock that have consumed the feed contaminated with aflatoxin B1 and B2 (Bakirci, 2001; Lopez *et al.*, 2001; Van Egmond, 1989). The hepatotoxic, genotoxic, mutagenic, carcinogenic, teratogenic, immunosuppressive and antinutritional effects of aflatoxins are well documented (Wangikar *et al.*, 2005; Williams *et al.*, 2004; Dichter, 1984). Aflatoxins are both acutely and chronically toxic for animals and

humans, and can produce dangerous illnesses including acute liver damage, liver cirrhosis, tumor induction and are also teratogen (Deshpande, 2002; Simon *et al.*, 1998).

International Agency for Research on Cancer (IARC) of WHO included AFB1 as primary and AFM1 as secondary groups of **carcinogenic** compounds (Cathey *et al.*, 1994; Dragacci *et al.*, 1995). In adult ruminants, exposure to aflatoxins can depress feed efficiency, immunocompetence and reproductive performance, as shown by studies with dairy cattle (Diekman & Green, 1992). The effects on feed efficiency presumably arise from impaired ruminal function, including reduced cellulose digestion, volatile fatty acid production and motility (Diekman & Green, 1992). In dairy cattle another problem arises from the transformation of AFB, to a related metabolite, aflatoxin M, which is secreted in the milk. Mycotoxins can increase the incidence of disease and reduce production efficiency in cattle (Coulombe, 1993; Joffe, 1986; Pier, 1992).

Mycotoxins can be the primary agent causing acute health or production problems in a dairy herd, but more likely, mycotoxins are a factor contributing to chronic problems including a higher incidence of disease, poor reproductive performance or suboptimal milk production. Recognition of the impact of mycotoxins on animal production has been limited by the difficulty of diagnosis. Molds can infect dairy cattle, especially during stressful periods when they are immune suppressed, causing a disease referred to as a mycosis. Ingestion of aflatoxins leads to substantial loss of productivity and degradation of meat quality in farm animals consuming contaminated feeds (Bonomi *et al.*, 1994).

Pathological effects vary between different mycotoxins and different animals. Ingestion of large amounts of toxin in a short period of time will cause acute toxicity leading to death while small doses in a prolonged length of time will result in chronic effects to the consumer. While healthy cows with an active immune system are more resistant to mycotic infections, dairy cows in early lactation are immune suppressed (Kehrli *et al.*, 1989). Aflatoxin lowers resistance to diseases and interferes with vaccine induced immunity in livestock (Diekman & Green, 1992). In beef cattle, Garrett *et al.* (1968) showed an effect on weight gain and intake with diets containing 700 ng/ml aflatoxin, but if increases in liver weights are used as the criteria for toxicity, 100 ng/ml would be considered toxic to beef cattle. Guthrie (1979) showed when lactating dairy cattle in a field situation were consuming 120 ng/ml aflatoxin, reproductive efficiency declined and when cows were changed to an aflatoxin free diet milk production increased over 25%. It must be also considered that young animals have been found to be more susceptible to AFB1 (and so probably AFM1) than adults.

The fungal growth and the formation of mycotoxins can occur in numerous vegetable species and may pose serious risks to human and animal health (Yannikuoris & Jouany, 2002). In favorable conditions to the development of toxigenous fungi, mycotoxins may be formed during any of the phases of production and transformation of food product. In particular, mycotoxins can be produced in plants infected during harvesting, during storage (and also transport), during the technological transformation and during preparation of food (Hussein & Brasel, 2001; Sweeney & Dobson, 1998).

They can be found in a diverse range of food and feed due to invisible spoilage in the field during plant growth, harvesting, storage and processing. It can be assumed that about 20% of food products (mainly of

plant origin) is substantially contaminated. Mold growth and the production of mycotoxins are usually associated with extremes in weather conditions leading to plant stress or hydration of feedstuffs, to poor storage practices, low feedstuff quality, and inadequate feeding conditions. Aflatoxins may be present in a large number of foods, *e.g.*, nuts, grain, groundnuts, dried fruit, figs, cereals (especially maize) and spices. Aflatoxin is more often found in corn, peanuts and cottonseed grown in warm and humid climates. Main sources of aflatoxins in feeds are peanut meal, maize and cottonseed meal. Although the highest concentrations are formed in food crops grown and stored in the warmer areas of the world, the international trading of these important commodities ensures that aflatoxins are not only a problem for producing countries but are also of concern for importing countries. Aflatoxin can be found in more temperate areas in some years as was seen in the drought year of 1988 when aflatoxin was found in 5% of corn grain in the Midwestern U.S. (Russell *et al.*, 1991). Contamination, either before or after harvest, of corn, peanuts, cereal crops, figs, oil seeds, spices and a long list of other commodities is a common occurrence (Ellis *et al.*, 1991; Miller, 1994).

Contamination of agricultural crops with aflatoxins is a worldwide problem not limited to developing countries, where both climatic and technological conditions stimulate aflatoxin formation.

Sources of aflatoxin contamination in animal feedstuffs may vary geographically. *A. flavus* and *A. parasiticus* colonize plants in the field, with the most risky geographical areas being those with tropical or subtropical climate, but they can also colonize products in post-harvest if not adequately dehydrated. The temperature growth range of these fungi is 12–48°C, but optimal conditions occur at 36–38°C. Aflatoxin production happens with temperatures between 20 and 30°C, and it seems that the higher limit is also the optimal one. Aflatoxin production is strongly correlated to kernel moisture; the way the mould penetrates the kernel and environmental conditions; in particular high temperatures and water stress. Cropping system can also play an important role in aflatoxin production; monoculture and the employment of hybrids unsuitable for the cultivation area with low resistance to insect attack (ears covered with thin bracts) are favourable factors for aflatoxin production.

In the surveys of Yoshizawa (1991), Strange (1991) and Shotwell (1991) evidence was presented for the widespread occurrence of aflatoxins in cereal grains, groundnut meal and cottonseed cake particularly in Uganda, Brazil, Nigeria and India. Although the incidence of contamination was generally low, some positive samples yielded unacceptably high levels.

The parameters affecting levels of AFM1 contamination in milk are the sources of animal feeds, ecologic and economic factors on the farm, and also farm management (Kuiper- Goodman, 1999).

Therefore, humans are potentially exposed to these metabolites and it is generally assumed that neither storage nor processing provides a reduction of AFM1 content (Unusan, 2006).

The forming of AFM1, metabolite of AFB1, occurs in the liver and it is secreted into milk in the mammary gland of dairy cows (Cathey *et al.*, 1994).

Many researchers reported that there was a linear relationship between the amount of AFM1 in milk and AFB1 in feed consumed by the animals. It is estimated that approximately 1–3% of aflatoxin B1 (AFB1)

initially present in animal feedstuff appears as AFM1 in milk, but this carryover rate has been shown to vary from animal to animal, day to day and also from one milking process to another (Van Egmond, 1989; Veldman, 1992; Caggioni & Pietri, 1999). Excretion of such toxins in bovine milk has been documented (Blüthgen *et al.*, 2004; Yiannikouris & Jouany, 2002) and their carryover to dairy produce represents a potential threat to human health. Therefore the presence of AFM1 in milk and milk products is considered to be undesirable (Galvano *et al.*, 1996; IARC, 1993; Masri *et al.*, 1974; Pietri *et al.*, 2003; Van Egmond, 1989).

On the other hand, AFM1 levels in milk show a seasonal variation and the toxin amount have differences in the products, which are produced from the toxin containing milk (Wood, 1991; Dragacci *et al.*, 1995).

1.3.1 Control of mycotoxin residues in milk

The most effective way of controlling aflatoxin M1 in the food supply is to reduce contamination with aflatoxin B1 of raw materials and supplementary feedstuffs for dairy cattle. Preventive measures must be applied to reduce fungal growth and aflatoxin B1 formation in agricultural commodities intended for use as animal feeds. In order to face the problem of aflatoxin M1 in milk and dairy products, it is necessary to focus the attention on the most sensitive steps of feedstuff production for lactating cows.

To prevent future aflatoxins outbreaks it is needed to communicate about the potential risk deriving from unsuitable farming managements that could lead to the development of contaminated feeds and foods.

When fungicides are used effectively to control fungal diseases of crop plants, then this risk is minimised. It is worthy of note that a number of insecticides are also effective in reducing or eliminating fungal proliferation and mycotoxin production. Much attention is now being given to breeding lines of cereal plants that are resistant to fungal colonisation and disease (Brown *et al.*, 1995; Campbell & White, 1995).

These studies show that AFB contamination of grain was generally reduced in maize hybrids resistant to ***Aspergillus*** ear rot. In the case of aflatoxin-contaminated oilseeds, specific detoxification procedures are commercially available in a number of countries (Park *et al.*, 1988). Ammoniation of contaminated meals appears to be the method of choice, involving treatment with either ammonium hydroxide or gaseous ammonia at high temperatures and pressure as in commercial feed mills or at ambient temperature and low pressure for small-scale operations. If the ammoniation reactions are allowed to proceed to completion, the detoxification process is irreversible and aflatoxin contamination is virtually eliminated (Phillips *et al.*, 1994). Ammoniation inactivates aflatoxins by hydrolysis of the lactone ring, which is followed by further breakdown. Ammoniation has been used in North America, Europe, and Africa on crops including maize, cottonseed, and peanut meal (Park *et al.*, 1988; Bailey *et al.*, 1994). Following detoxification by ammoniation, the treated crop products are nutritionally valuable for domestic animals, but are not suitable for human consumption.

Some additives are beneficial in reducing mold growth and therefore mycotoxin formation. Ammonia, propionic acid, sorbic acid and microbial or enzymatic silage additives are shown to be at least partially effective at inhibiting mold growth.

Care should be taken to ensure that high moisture grains are stored at proper moisture contents and in a well maintained structure. Grains or other dry feed, such as hay, should be stored at a low moisture content (<14%) below which molds do not readily grow, and then protected to remain dry.

1.3.2 Maximum Residue Limit (MRL)

Mycotoxins are regulated in more than 77 countries worldwide (FAO, 1997), while regulations vary from country to country on the type of mycotoxin, matrix (type of food or feed) as well as the maximum allowed level. Contamination limits of 2 µg/kg aflatoxin B1 allowed in food products such as cereals, peanuts, pistachios and figs marketed in Europe are five times lower than those in the US. However, not only legislative limits have been regulated, but also requirements for laboratories that are involved in the official control of foodstuffs as well as for sampling and analysis methods have been defined.

The European Community and *Codex Alimentarius* prescribe that the maximum level of AFM1 in liquid milk and dried or processed milk products should not exceed 50 ng/kg (*Codex Alimentarius* Commissions, 2001). However, according to US regulations the level of AFM1 in milk should not be higher than 500 ng/kg (Stoloff *et al.*, 1991). In Austria and Switzerland the maximum level is further reduced to 10 ng/kg for infant food commodities (FAO, 1997). There are thus differences in maximum permissible limit of AFM1 in various countries (Van Egmond, 1989), and many including Iran and Pakistan, which have not imposed any legal limit for aflatoxin M1 in dairy products so far.

Regulatory limits throughout the world are influenced by considering each countries conditions, and may vary from one country to another (Chen & Gao, 1993; Stahr *et al.*, 1990; Stoloff *et al.*, 1991; Van Egmond, 1989). Thus, strict regulatory limits for these compounds are currently in force in developed countries, and accurate monitoring analysis has been initiated.

1.3.3 Sampling and analysis of AFM1

Therefore the current regulation on aflatoxins will serve here as an example: with the introduction of Regulation 466/2001/EEC, legislative limits for aflatoxins were directly linked (with reference to Directive 98/53/EEC) to the sampling method and to requirements on analytical methods to be used for enforcement of food control (European Commission, 2001).

Concerning the acceptance of analytical methods, several approaches exist at European Community level. One strategy is the draft of an explicitly defined method as a reference method. In this case the directive contains detailed information on the laboratory equipment and material to be used for analysis (method description), as it has been done in the past for other contaminants/ingredients and quality standards for food additives by Directive 81/712/EEC.

Thus a horizontal definition concerning the performance criteria of methods, as it has been done in Directive 98/53/EEC for aflatoxins, allows the use of different state-of-the-art methods (method principles). In addition, with reference to Directive 85/591/EEC, the frameworks for conduction of collaborative trial studies for the elaboration of the method performance parameters have to be in compliance with

internationally accepted protocols. Since the discovery of mycotoxins, several methodologies for their determination have been developed. Methods routinely used nowadays are mainly based on either thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) or enzyme linked immuno sorbent assay (ELISA). Immunochemical assays are rapid, simple, specific, sensitive, and have become the most common quick methods for the routine analysis of mycotoxins in food and feed materials (Magliulo *et al.*, 2005; Rodriguez Velasco *et al*, 2003; Stroka & Anklam, 2002; Thirumala-Devi *et al.*, 2002). The aflatoxin content in positive samples can later be confirmed by HPLC analysis (Markaki & Melissari, 1997).

2. THESIS OBJECTIVES

The aims of the present thesis were to assess the situation over the presence of contaminants in milk for human consumption in Kosovo. The specific aims were as follows:

1. To evaluate the occurrence of contaminant residues in milk samples obtained from different collection sites in the state of Kosovo

2. To validate screening tests available at official laboratories for the analysis of contaminants in milk.

3. Facilitating the organization and development of national monitoring and surveillance program and national food chain safety policy to provide the scientific data for risk analysis.

4. Supporting the Institutions of Kosovo, strengthening the official control service under the Kosovo Food veterinary Agency to ensures a high level of public health and consumer confidence in food of animal origin.

5. Facilitating KFVA and the laboratory staff in participating in proficiency testing as integral part of good laboratory practice.

6. Assisting in development of Food chain safety and the protection of consumer interests governmental organizations, professional associations, international trading partners and trade organizations.

3. MATERIALS AND METHODS

3.1 Facility and equipment

Research has been conducted at the Kosovo Food and Veterinary Agency – Food Safety Laboratory in Prishtina. The research lasted three years from 2008 to 2010, 2000, milk samples were collected from different parts of Kosovo and at different season time in the year.

3.2 Location of milk collection point sampled

This research has been targeted geographically in six regions of Kosovo, during a whole year, systematically two times per month in two collection points in the region.

The sampling of raw milk was territorially programmed in such a way that the results could describe the level of milk contamination in the whole territory of Kosovo.

The samples of raw milk were collected from six major regions in Kosovo (Prishtina, Gjilan, Mitrovica, Peja, Gjakova, Prizren) Figure 1. Our sampling was carried out in different periods of the year- spring, summer, autumn and winter. The sampling included areas of intensive and extensive milk production according to the milk route collection.

Figure 1: Location of milk sampling in the major regions in Kosovo

Samples of raw milk were collected at individual farms or at two milk collection points (MCP), in each region two times per month; samples of UHT milk were collected from retail markets all year long.

All of the samples were analyzed with Delvotest SP ≤24 h after milking from cow. In 2008, a total of 161 samples were collected, 144 raw milk samples from two milk collection points (MCP) and 17 samples from individual cows directly at farms.

In 2009, a total of 1015 samples were collected, 826 came from MCP, 150 from individual cows and 39 samples of UHT milk from retailers, whereas in 2010, 719 samples were analysed, milk from individual

farms (54), milk from collection points (582) and UHT milk (30) from retail markets in Prishtina City. Our research examined totally 1895 samples of milk. (Figure 2)

Samples of raw milk (500 ml) were taken from the plants' raw milk tanks with raw milk jar samplers, transported at 2– 4 °C in an icebox before arrival in the laboratory of the Kosovo Veterinary Laboratories, and screened within 24h for presence of antimicrobials by DELVOTEST SP (DSM Food Specialities, Dairy Ingredients, Delft, The Netherlands).

Antibiotic-free bovine milk (blank sample to be used as negative control and as positive control –if spiked with a known amount of antibiotics) was collected from one milking cow not treated with any drugs for the previous 3 months. In 2008, all samples reacting positive on screening test (DELVOTEST SP) were analyzed for confirmatory purposes by HPLC with diode array detector in the case of tetracyclines and by LC-MS in the case of sulphonamides and β-lactams at the Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy. In 2009 and 2010, all the positive samples at the DELVOTEST SP were checked again with the new SNAP TEST (IDEXX Laboratories, Westbrook, USA) specific for the 3 different drug classes: Penicillins, Tetracyclines and Sulphonamides. Only the samples that were confirmed by the second screening test underwent the same extraction and chemical confirmation procedure reported for the 2008 milk samples.

Figure 2. Different types of milk samples prepared for the analysis

3.3 Screening methods

Residues of antibiotics in milk were determined using the standard microbiological methods called Delvotest SP (DSM Food Specialities, Dairy Ingredients, Delft, The Netherlands) and SNAP test (Idexx Laboratories Inc., Westbrook, ME, USA) which is an enzyme-linked, receptor binding assay. The screening test (DELVOTEST SP AND SNAP TESTS) to evaluate the presence of veterinary drugs in raw milk were carried out at the Institute of Food Safety Control of the Kosovo Food and Veterinary Agency.

3.3.1 Delvotest “SP” Microbial Test

The commercial Delvotest SP (manufactured by DSM Food Specialties, Delft, The Netherlands) was carried out according to the instructions of the manufacturer. The assay procedure included these steps: one ampoule and label for identification for each milk sample to be tested should be taken and placed in the special holes

in the Delvotest box. The ampoule should be than opened by punching a small hole in the aluminum foil with for example the blunt end of the syringe (without removing foil from ampoules). A fresh disposable pipette should be placed onto the syringe for each milk sample to be tested. The milk sample (0.1 ml) will be added by depressing the plunger of the syringe completely and insertion the tip of the pipette approx. 1 cm into the milk sample and allow the plunger slowly to return to the start position.

The milk sample in the pipette (0.1 ml) should be transferred completely to the correspondingly labeled ampoule. This is done by depressing the plunger slowly, adding the milk straight onto the agar. Ampoule(s) than should be placed in a preheated dry incubator at 64°C ± 0.5°C. Incubation of the ampoules last for 3 hours. After incubation of the ampoules (3 hours / 64°C) the ampoules should be withdrawn from incubator and the test results should be read. The results should be read from the lower 2/3 of the agar. A yellow colour indicates the **absence** of antibacterial substances in the related milk sample at a concentration at or above the test's detection limit. A yellow/purple colour indicates the **presence** of antibacterial substances close to the test's detection limit. A purple colour indicates the **presence** of antibacterial substances in the related milk sample at or above the test's detection limit. Figure 3 shows the process of analysis and change of color when positive sample is detected. By applying the Delvotest SP, milk was tested for residues of the following antibiotic classes: penicillins, sulphonamides and tetracyclines.

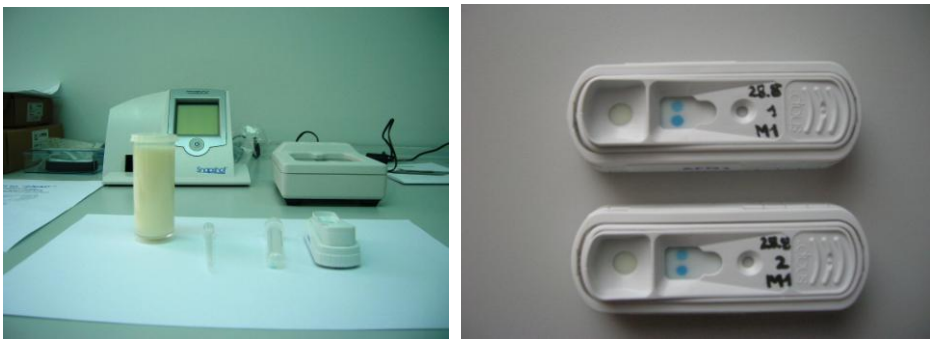
Figure 3 Samples and analysis of milk with Delovtest SP

3.3.2 IDEXX SNAP test

The Snap tests used to detect presence of antimicrobials (beta-lactams, tetracyclines and sulphonamides), were SNAP Beta-lactamtest kit, SNAP Tetracyclines Test Kit and Sulphamethazin Test Kit. The SNAP test takes 10 minutes to be completed. Since in our studies all positive samples were incriminated with beta-lactam antibiotics, details of the working procedures for SNAP Beta-lactam test will be explained below. SNAP new beta lactam test (Idexx Laboratories Inc., Westbrook, ME, USA) is an enzyme-linked, receptor binding assay in which β-lactams are captured by a binding protein on a solid support adsorbent matrix housed in a moulded plastic unit. SNAP residues test consists of three components: SNAP device, pipette, sample tube. Using this test, penicillin can be detected in the amount of 4 μg/kg, ampicillin or amoxicillin in the amounts of 10 μg/kg, cephapirin 8 μg/kg, and ceftiofur 50 μg/kg. The SNAP test utilizes a beta-lactam receptor protein conjugated to an enzyme. The assay procedure includes three simple steps with a total assay

time of about 10 minutes for a sample. In the first step of test, calibrated amounts of milk and conjugate are mixed and incubated in a test tube, placed in a heating block (5 min, 45 ± 5°C). The enzyme conjugate binds with beta-lactams present in the milk sample. The mixture is then transferred to the sample well of the SNAP device (plastic unit containing sample and control spots on filter paper strip) where the sample is allowed to migrate on a filter paper strip until it passes to the test spot. Test spots are coated with beta-lactam antibiotic. Any free receptor will be captured at this spot, whereas the receptor protein that interacts with free beta-lactams in the sample will not. The substrate is released and reacts with the enzyme attached to the captured receptor protein and a colour develops at the test spot. The results are read either visually or instrumentally (using reflectance) to provide the numerical interpretation of the visual result. The samples are declared positive or negative on the basis of the comparison of the intensity of the colour development between the sample and control spots on the SNAP test. If the color of the test spot is weaker than that of the control spot, the result is interpreted as positive. Preparation of sample and the Snap test device after sample was analysed may be seen in Figure 4.

Figure 4 Samples and analysis of milk with Snap test



3.3.3 ELISA Penicillin Test

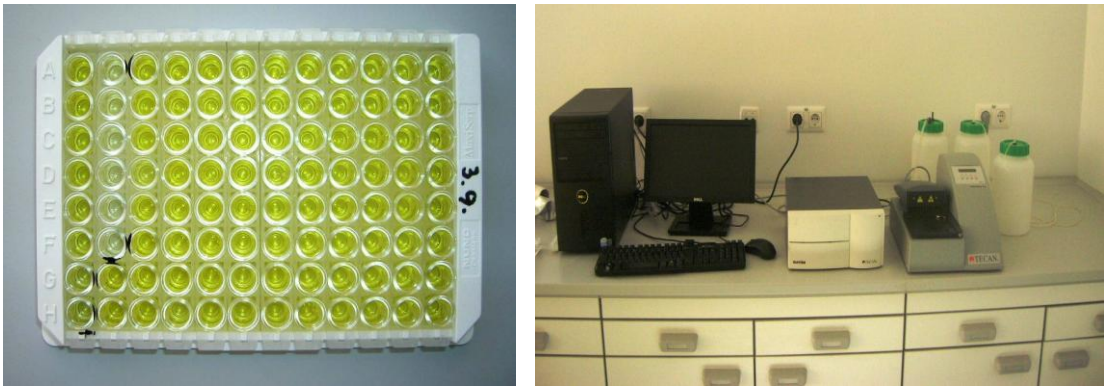
96 Tests Enzyme Immunoassay for the Quantitative Determination of Penicillin in Milk and Shrimps Immunolab GmbH, Germany Analysis of b-lactams in samples by the ELISA. The quantitative ELISA kit Penicillin in milk (immunolab GmbH, Germany) was stored at 2–8°C. Before its use the kit was left for 2 h at room temperature to bring it to room temperature. The KIT was used according to the manufacturer's instruction (Romer Labs, 2005) as follows.

Into Penicillin-antibody-coated micro titer plate 100 µL ready-to use standards or prepared samples in duplicate were pipetted into each well (100 µL/well of standard) and immediately 50 µL of penicillin antibody was added into each well. The plate containing the samples was covered with a plastic foil and incubated at room temperature for 60 min using a microtiter plate shaker (or 90 minutes without shaker).

Following a washing step with washing solution (supplied with the KIT), 100 µL of conjugate(anti-mouse-IgG-HRP) was added to the wells, and the plate was covered with a plastic foil and incubated again at room temperature for 60 min on a microtiter plate shaker (or 90 minutes without shaker). The plate was washed with the washing solution in order to remove the unbound conjugate. A 100 µL of substrate solution was added into the wells and the reaction was allowed to proceed in the dark for 20 min at room temperature, at

the end of which a blue colour is developed. The reaction was stopped by adding 100 µL of stop solution (0.5 M H_2SO_4) to the wells, and the colour changed from blue to yellow. The absorbance was measured at 450 nm in Multiskan Ascent ELISA Plate Reader (Tecan Reader Tecangroup, SWITZERLAND) as it may be seen in Figure 5. The colour is stable for 30 minutes, the concentration of penicillin is indirectly proportional to the colour intensity of the test sample. The log–logit AFM1 sheet supplied with the KIT was used to generate a standard curve and to calculate the concentration of penicillin in the samples.

Figure 5 Test Plate after analysis, plate analysed by Tecan reader



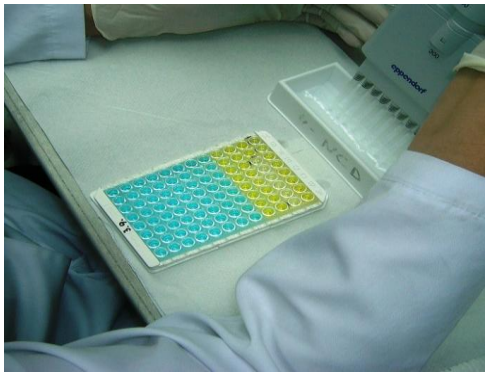
3.3.4 Enzyme immunoassay for the detection of Aflatoxin M1 in milk

I'screen AFLA M1 milk is a kit prepared for an immunoenzymatic assay for the quantitative analysis of aflatoxin M1. The kit contains the procedure and the materials sufficient for 96 determinations (code MA440) or 48 determinations (code MA441) including standards. A microtiter plate photometer, or a strip photometer is required. Analysis of AFM1 in samples by the competitive ELISA The quantitative ELISA kit AgraQuant Aflatoxin M1 (I'screen AFLA M1 milk, Tecna Srl, Italy) was stored at 2–8°C. Before its use the kit was left for 2 h at room temperature to bring it to room temperature. The KIT was used according to the manufacturer's instruction (I'screen AFLA M1 milk, Tecna Srl, Italy) as follows:

Analysis of AFM1 in samples by competitive ELISA The quantitative analysis of AFM1 in samples was performed by competitive ELISA using a AFM1 test kit (I'screen AFLA M1 milk, Tecna Srl, Italy). The AFM1 standards and test samples (100 µl) in duplicate were added to the wells of micro-titer plates precoated with antibodies for AFM1 and incubated at room temperature in dark for 45 min. After the washing step, AFM1- Horseradish Peroxidase conjugate (supplied with the kit) was added (100 µl) to the wells and the plate was incubated again for 15 min at room temperature in dark. The unbound conjugate was removed during washing. The enzyme converts the colourless chromogen into a blue product during the third incubation. The color change is shown in the Figure 6. Subsequently, the reaction was stopped by 50 µl of stop solution which were added to the wells and mixed thoroughly with rotatory motion for a seconds. The addition of the stop reagent leads to a color change from blue to yellow and the absorbance was measured

at 450 nm in Spectramax ELISA plate reader (Molecular Devices Corporation,CA,USA). The colour development is inversely proportional to the Aflatoxin M1 concentration in the sample.

Figure 6 Milk samples for AFM1 testing and the analysis of the samples



3.4 Confirmatory analytical methods

The chemical confirmatory methods (HPLC-DAD for tetracyclines and LC-MS for sulphonamides and β-lactams) were carried out at the Dipartimento di Sanità pubblica, Patologia comparata e Igiene veterinaria of the Padova University and those for Tetracycline at the Istituto Zooprofilattico Sperimentale delle Venezie Padova, in Italy.

3.4.1 Tetracycline

The extraction method was an adaptation of that described by (Cristofani *et al.*, 2009). Briefly, 3 g of milk were extracted with 20 ml of succinic acid 0.1 M pH 4 and with 20 ml of methanol by mechanical shaking for 30 seconds and by sonication in an ultrasonic bath for 10 minutes. Liquid phase was separated from solid residue by centrifugation at 5000 g for 10 minutes. Extraction was repeated a second time by adding 10 ml of succinic acid 0.1 M pH 4 and 10 ml of methanol. After centrifugation at 5000 g for 10 minutes, and extracts were re-unified and purified through Methal Chelate Affinity Chromatography (MCAC) columns activated with 6 ml of distilled water, 3 ml of $CuSO_4$ 10 mM and 4 ml of distilled water. After extracts loading, column were washed with 2 ml succinic acid 0.1M pH 4, 2 ml distilled water, 2 ml methanol and 2 ml distilled water, finally elution was performed by application of 8 ml McIlvaine buffer. The eluate was purified by OASIS HLB SPE columns (60 mg, 3ml) previously activated with 3 ml of methanol, 3 ml HCl 1N, 3 ml of distilled water and washed with 3 ml distilled water. Elution was achieved by 5 ml of methanol. Solvent was evaporated to dryness under N_2 stream and the residue was re-dissolved with 0.5 ml of oxalic acid 0.01 M before injection in HPLC-DAD.

Tetracycline determination was performed by HPLC-DAD using the chromatographic condition briefly reported: Chromatografic column: Ascentis Express C18 2.7μm 150 x 4.6 mm (Supelco); injection volume:

20 µl. Auto-sampler temperature: 5°C. Column temperature: 30°C. Detection system: UV-DAD with monitoring wavelength: 355 nm, and UV spectrum range: 210-450 nm. The gradient elution conditions are reported in table 5 (see below). Quantification was performed against external calibration in pure solvent, as matrix effects were negligible, and quantified concentrations were corrected for recovery.

Table 5: HPLC-DAD gradient elution conditions for tetracyclines

Time [min]	% Solvent A	% Solvent B	% Solvent C	% Solvent D	Flow [ml/min]	Curve
0 - 1	0.0	9.0	70.0	21.0	0.6	1
1 - 6	0.0	22.0	70.0	8.0	0.6	6
6 - 12	0.0	22.0	70.0	8.0	0.6	6
12 - 13	0.0	9.0	70.0	21.0	0.6	6
13 - 18	0.0	9.0	70.0	21.0	0.6	6
B = acetonitrile **C** = Ossalic acid 0.01 M **D** = Methanol						

3.4.2 Sulphonamides

5 g of milk were extracted with 15 ml of ethyl acetate in presence of 8 g of $NaSO_4$ anhydrous by mechanical shaking for 15 minutes. Organic phase was separated from solid residue by centrifugation at 6000 g for 10 minutes. Extraction was repeated a second time by adding 15 ml of ethyl acetate. After centrifugation at 6000 g for 10 minutes, extracts were re-unified and evaporated to dryness under N_2 stream at 60°C. The residue was re-dissolved with 10 ml of HCl 0.1 N and defatted with 2 x 5 ml of n-hexane. After centrifugation at 4000 g for 5 minutes, the upper organic layer was eliminated and the aqueous phase purified through Strata XC SPE columns (200 mg, 3ml) previously activated with 5 ml of methanol, 5 ml HCl 0.1N and washed with 4 ml HCl 0.1N, 4 ml methanol. Elution was achieved by 5 ml of ammonium hydroxide solution max 33%/methanol 30/70 v/v. Solvent was evaporated to dryness under N_2 stream at 60°C and the residue was re-dissolved with 0.5 ml of formic acid 0.05 M/ acetonitrile 85/15 v/v. The final extract was diluted 1/10 with mobile phase before HPLC-MSMS analysis. Sulphonamides determination was performed by HPLC-MSMS according to the following chromatographic conditions: chromatografic column: Phenyl X-Terra 3.5µm 100 x 2.1mm (Waters) and spectrometric conditions reported in table 6. Injection volume: 5 µl. Auto-sampler temperature: 5°C. Column temperature 30°C. The elution was done at gradient condition reported in table 2 at the operative conditions of the analytical detection MS/MS reported in table 7.

Table 6: LC-MS gradient elution conditions for sulphonamides

Time [min]	% Solvent A	% Solvent B	% Solvent C	% Solvent D	Flow ml/min	Curve
0 - 1	95	0	0	5	0.25	1
1 - 15	70	0	0	30	0.25	6
15 - 16.5	70	0	0	30	0.25	6
16.5 - 17	10	0	0	90	0.25	6
17 - 19	10	0	0	90	0.25	6
19 - 20	95	0	0	5	0.25	6
20 - 28	95	0	0	5	0.25	6
A = formic acid 0.05 M **D** = acetonitrile						

Table 7: Detection system: MSMS analyzer. Operative conditions for sulphonamides

Analyte	MSMS transition (Collision energy)	
Sulfacetamide	215 > 156 (10)	215 > 108 (19)
Sulfadiazine	251 > 156 (15)	251 > 108 (25)
Sulfapyridine	250 > 156 (16)	250 > 108 (25)
Sulfathiazole	256 > 156 (15)	256 > 92 (25)
Sulfamerazine	265 > 156 (16)	265 > 172 (15)
Sulfamethazine	279 > 204 (17)	279 > 156 (18)
Sulfamethoxypyridazine	281 > 156 (19)	281 > 108 (29)
Sulfamonomethoxine	281 > 156 (19)	281 > 108 (29)
Sulfachlorpyridazine	285 > 156 (15)	285 > 108 (25)
Sulfadoxine	311 > 156 (19)	311 > 108 (29)
Sulfamethoxazole	254 > 156 (15)	254 > 108 (25)
Sulfisoxazole	268 > 156 (14)	268 > 113 (15)
Sulfadimethoxine	311 > 156 (20)	311 > 108 (30)
Sulfaquinoxaline	301 > 156 (18)	301 > 108 (29)
Ionisation mode	ESI +	
Capillary voltage	2.90 kV	
Cone	38 V cone gas flow 50 l h^{-1}	
Source temperature	125 °C	
Desolvation temperature	325 °C	

3.4.3 Beta-lactams

Aliquots of 5 g of milk were extracted with 10 ml of acetonitrile by mechanical shaking for 10 minutes. Organic phase was separated from solid residue by centrifugation at 4000 g for 5 minutes. Extraction was repeated a second time by adding 10 ml of acetonitrile. After centrifugation at 4000 g for 5 minutes, extracts were re-unified and evaporated to 0.5 ml under N_2 stream at 50°C and 4 ml of phosphate buffer 0.05 M pH 7.5 were added. The extract was defatted with 5 ml of n-hexane. After centrifugation at 4000 g for 5 minutes,

the upper organic layer was eliminated and the aqueous phase purified through OASIS HLB SPE columns (60 mg, 3ml) previously activated with 2 ml of methanol, 2 ml of distilled water and 2 ml of phosphate buffer 0.05M pH 7.5 and washed with 3 ml of phosphate buffer 0.05 M pH 7.5 and 1 ml of distilled water. Elution was achieved by 5 ml of acetonitrile. Solvent was evaporated to dryness under N_2 stream and the residue was re-dissolved with 0.5 ml of Ammonium formiate 0.05M pH 7.5 / acetonitrile 90/10 v/v. A volume of 10 µl was injected into HPLC-MSMS for beta-lactams determination. For the chromatografic elution a column X Bridge C18 3.5µ, 2.1 x 150 mm (Waters) was set at the temperature 30°C and the Autosampler temperature was 5°C. The gradient elution conditions and spectrometric conditions were reported in table 8 and 9 respectively.

Table 8: LC-MS gradient elution conditions for beta-lactams

Time [min]	% Solvent A	% Solvent B	% Solvent C	% Solvent D	Flow [ml/min]	Curve
0 – 1	90	10	0	0	0.25	1
1 – 6	65	35	0	0	0.25	6
6 – 12	50	50	0	0	0.25	6
12 – 14	25	75	0	0	0.25	6
14 – 15	25	75	0	0	0.25	6
15 - 15.50	90	10	0	0	0.25	6
15.50 – 24	90	10	0	0	0.25	6

A: 0.1% Formic acid in distilled water **B**: 0.1% Formic acid in acetonitrile

Table 9: Detection system: MS/MS analyzer. Operative conditions for beta-lactams.

Analyte	MSMS transition (Collision energy)	
Amoxicillin	366 > 349 (7)	366 > 114 (20)
Amoxicillin-d4	370 > 353 (8)	
Cefapirin	424 > 292 (13)	424 > 152 (24)
Cefalonium	459 > 337 (9)	459 > 152 (18)
Ampicillin	350 > 192 (15)	350 > 106 (18)
Ampicillin –d5	355 > 111 (18)	
Cefalexin	348 > 191 (6)	348 > 174 (15)
Cefalexin – d5	353 > 158 (7)	
Cefazolin	455 > 323 (10)	455 > 156 (14)
Ceftiofur	524 > 210 (20)	524 > 285 (18)
Ionisation mode	ESI +	
Capillary voltage	3.20 kV	
Cone	35 V	
Source temperature	125 °C	
Desolvation temperature	325 °C	
Desolvation gas (nitrogen) flow	900 l h^{-1}	
Cone gas flow	50 l h^{-1}	
Analyte	**MSMS transition (Collision energy)**	
Cefuroxime	423 > 318 (7)	423 > 207 (12)
Cefaperazone	644 > 115 (30)	644 > 188 (20)
Penicillin G	333 > 192 (10)	333 > 289 (6)
Penicillin G-d7	340 > 199 (10)	
Penicillin V	349 > 208 (8)	349 > 114 (18)
Penicillin V-d5	354 > 213 (9)	
Oxacillin	400 > 259 (12)	400 > 356 (6)
Cloxacillin	434 > 293 (11)	434 > 390 (8)
Nafcillin	413 > 272 (12)	413 > 369 (8)
Dicloxacillin	468 > 327 (11)	468 > 424 (7)
Ionisation mode	ESI -	
Capillary voltage	2.80 kV	
Cone	35 V	
Source temperature	125 °C	
Desolvation temperature	325 °C	
Desolvation gas (nitrogen) flow	900 l h^{-1}	
Cone gas flow	50 l h^{-1}	

4. RESULTS

4.1 Residue of antibacterials in milk

4.1.1 Results of screening methods

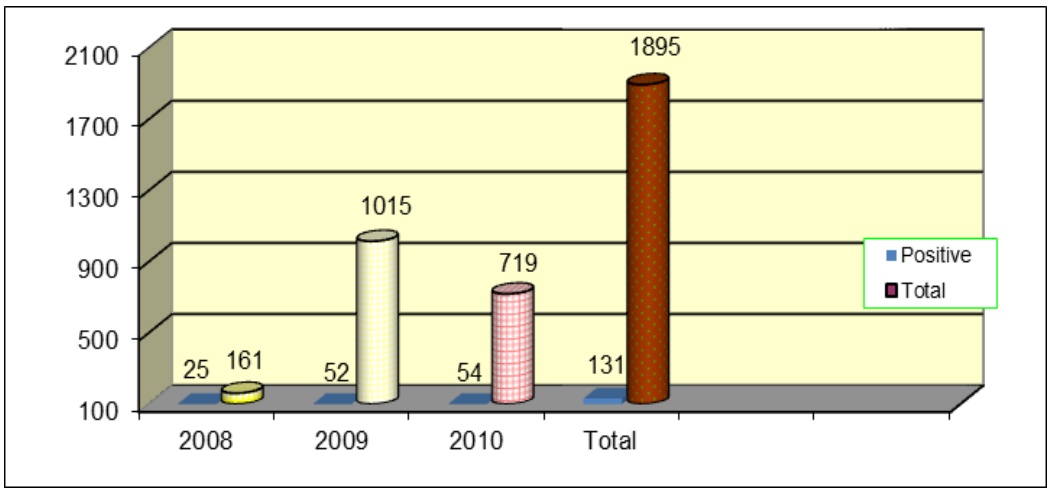
The qualitative examination of antibiotic residues in 1895 milk samples, during a three year period (2008 to 2010), led to the identification of 131 positive samples (6.91%), 5 dubious samples (0.27%) and 1759 negative samples (92.82). Total number of analyzed samples for residues of antibiotics during year 2008-2010 is shown in table 10.

Table 10 Total No of examined samples during 2008-2010

Raw milk	No. of samples	No. of positive samples	No. of negative samples	No. of ambiguous samples
2008	161	25 (15.52%)	136 (84.47%)	0
2009	1015	52 (5.12%)	960 (94.58%)	3 (0.30%)
2010	719	54 (7.51%)	663 (92.21%)	2 (0.28%)
Total	**1895**	**131 (6.91%)**	**1759 (92.82%)**	**5 (0.27%)**

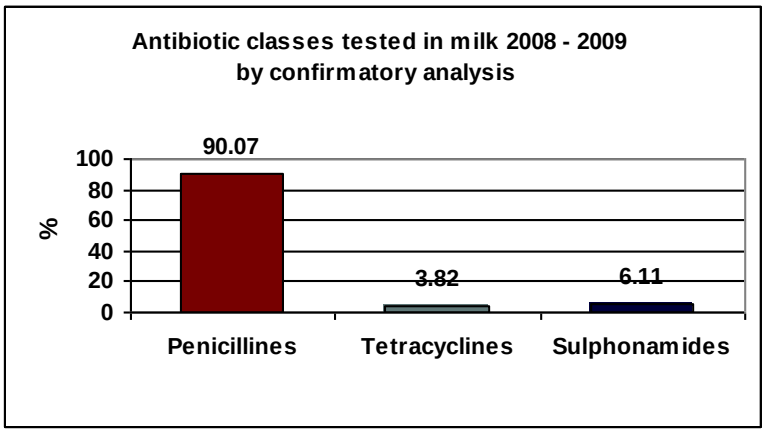
In 2008, 25 out of 161 samples were positive (15.52%) in 2009, 52 out of 1015 samples were positive (5.12%) and in 2010, 54 out of 719 samples were positive (7.51%). The total number of positive samples found the study is shown in figure 7.

Figure 7. Number of tests and positives for milk, 2008-2010



The main antibiotic families were betalactams (in 90.07% of samples) in the range 0.2-1973.4 µg/kg, tetracyclines (in 3.82% of samples) in the range 20.0-43760 µg/kg and sulphonamides (in 6.11% of samples) in the range 0.3-21 µg/kg as it can me seen in figure 8.

Figure 8 Antibiotic classes tested in milk during 2008-2009 by confirmatory analysis



In 2008, one hundred and sixty-one milk samples (161) from milk from individual farms (17), milk from collection points (144) were analyzed, and UHT milk (42) were collected from different retail markets and brands within Pristine City. Results are shown in table 11.

Table 11: Screening data obtained by Delvotest SP of 161 milk samples collected in 2008

Milk	2008	Negative samples	Positive samples	Ambiguous samples
Milk from individual farms	17	0	17	0
Milk from collection points	144	136	8	0
UHT milk				0
Total	**161**	**136**	**25**	**0**

In 2009 the research materials consisted of 1015 milk samples, milk from individual farms (150), milk from collection points (826) and UHT milk (39), from retail markets (local and foreign producers), from six different major areas of Kosovo. In 2010, 719 samples were analysed, milk from individual farms (54), milk from collection points (635) and UHT milk (30) from retail markets in Prishtina City, data are shown in table 12.

Table 12: Screening data obtained by Delvotest SP of 1734 milk samples collected from different sites in 2009-2010

Milk surveillance during 2009-2010								
	2009			**2010**				
Milk samples	samples	negative samples	ambiguous samples	positive samples	Samples	negative samples	ambiguous samples	positive samples
individual farms	150	140	0	10	54	52	*2*	*0*
collection points	826	785	2	41	635	582	*0*	*53*
UHT milk	39	38	0	1	30	29	*0*	*1*
Total	***1015***	*966*	*2*	*52*	***719***	*663*	2	54

In this study 895 milk samples were analyzed by Delvotest SP. All positive samples were analysed with further two screening tests to better evaluate the contamination of antibiotics. In 2009 Milk samples tested by Delvotest SP (Tecna srl, Trieste, Italy) were 1015, and 52 samples were tested with SNAP Afla M1 test (IDEXX Laboratories, Westbrook, Maine USA). In 2010 total of 719 milk samples were tested by Delvotest SP, 37 samples were tested with SNAP Afla M1 test (IDEXX Laboratories, Westbrook, Maine USA), and with Enzyme-linked immunosorbent assay (ELISA, Immunolab, Kassel/Germany) test 37 positive samples. Screening data obtained by Delvotest SP, Snap Test of 1015 milk samples collected in 2009/2010 are shown in table 13.

Table 13: Screening data obtained by Delvotest SP, Snap Test of 1015 milk samples collected in 2009/2010

Screening data obtained by Delvotest SP or Snap Test								
	2009				**2010**			
Analytical test	**samples**	**negative samples**	**ambiguous samples**	**positive samples**	**samples**	**negative samples**	**ambiguous samples**	**positive samples**
Delvotest SP	**1015**	**963**	**0**	**52**	**719**	**663**	***2***	***54***
New Snap beta lactam test	**52**	**0**	**2**	**40**	**54**	**0**	***0***	***54***
Snap tetracycline test	**52**	**52**	**0**	**0**	**54**	**54**	***0***	***0***
Snap test Sulphameth.	***52***	***52***	***2***	***0***	***54***	***54***	**0**	**0**
ELISA beta lactam test	***0***	***0***	***0***	***0***	***37***	***0***	**19**	**17**

4.1.2 Results of ELISA Penicillin test

The test Elisa adpted had a detection range between 3-950 ng/L was used to check 37 samples which were positive on Delvotest SP and Snap Betalactam test. Elisa test has confirmed 17 samples positive in the range from 10-950 ng/L, as shown in table 14.

Table 14: Concentrations obtained by ELISA beta lactam for positives 2010

No. of positive samples	Range (ppb)
0	3-10 ng/L
4	10-40 ng/L
4	40-100 ng/L
3	100-200 ng/L
3	200-400 ng/L
3	400-950 ng/L

4.1.3 Results of Confirmatory analysis

The analytical methods applied to 80 sample extracts (reacted positive at screening methods) confirmed the presence of tetracyclines (oxytetracycline, tetracycline) by LC-DAD in 5 samples (3 of them with concentration > MRL), the presence of sulfonamides (sulfadiazine, sulfathiazole, sulfamethazine and sulfamethaxazole) by LC-MS in 8 samples (all of them with concentration << MRL) and the presence of β-lactams (amoxicillin, penicillin G, cefazolin and cloxacillin) by LC-MS in 46 samples (21 of them with concentration > MRL) see table 15.

Table 15 Concentrations obtained by confirmatory methods 2008-2009

TETRACYCLINES **EU MILK MRL: 100 ppb**	**Range (ppb)**
Oxytetracycline	20 - 43760
Tetracycline	1030
SULPHONAMIDES **EU MILK MRL: 100 ppb**	**Range (ppb)**
Sulfamethazine	2.3 - 19
Sulfamethoxazole	0.3 - 0.8
Sulfathiazole	3.6 – 9.4
Sulfadiazine	1.7 – 21.0
β-LACTAMS	**Range (ppb)**
Penicillin G (EU MILK MRL: 4 ppb)	0.56 – 1973.4
Cefazolin (EU MILK MRL: 50ppb)	1.3
Cloxacillin (EU MILK MRL: 30 ppb)	0.48 - 542.0
Amoxicillin (EU MILK MRL: 4 ppb)	0.06 - 42.9
Ampicillin (EU MILK MRL: 4 ppb)	0.21 - 784.2

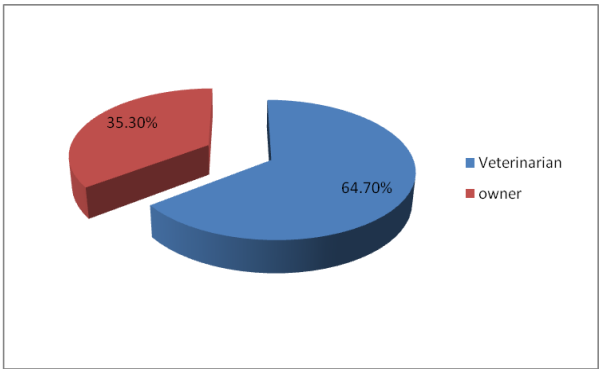
A study was conducted the first year of research (2008), and we analyzed a total of 161 samples of raw milk which were randomly obtained from several collaborating dairies. The samples were collected from June to October 2008, at the beginning and end of the month.

Information was collected in the farms were positive cases of antibiotic residues have been found and information was collected in regarding antibiotics used in dairy herds for disease prevention and treatment, determination of patterns of use of antibiotics by herd's size animal age group, animal breed, and determination of frequency of the administration of drugs by veterinarians and farmers for prevention and treatment.

In order of frequency, the most commonly used preventive antibiotic and sulphonamides were penicillins, tetracyclines, and sulphonamides, making up over 90 % of all antimicrobials used for disease treatment.

In the treatment of cattle in farms antibiotics were administered mainly by veterinarians in 64.70% of cases. Other than the veterinarian, antibiotics were administered primarily by the owner/manager on 35.30% of the cases. This is shown in figure 9. The main route for drug administration was intra mammary i.m., application 76.47%. Main breed treated with antibiotics and thus with positive test results on residues was Holstein Frisian breed with 47.05%. The Most frequent age of affected animals were 5 years with 41.17%.

Figure 9 Administration of antibiotics in cattle by Veterinarians/Owners



In mastitis, penicillins, tetracyclines, sulphonamides made up to 95 % of all antimicrobial preventive administration. The largest numbers of treatment drugs for cows were used for respiratory tract conditions, mastitis, gastrointestinal tract conditions and breeding problems (including metritis) in cows (table 16).

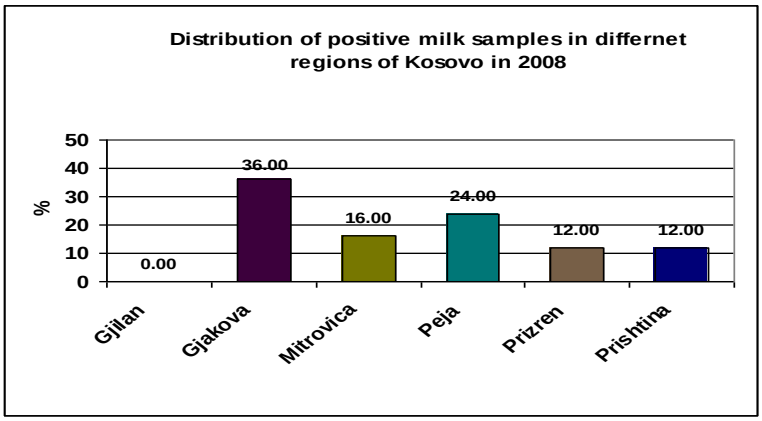
Table 16 – Antibiotics and Sulfonamides used for disease prevention for top four diseases in cows.

Drug type	Mastitis	Gastrointestinal tract disease	Breeding	Respiratory tract disease
Penicillins	4	1	1	5
Tetracyclines	1	2	0	2
Sulphonamides	1	1	1	1
Total	6	4	2	8

Among all the disease conditions, pneumonia and mastitis were the cases where various classes of antibiotics were used (table 16). In lactating cattle, clinical mastitis was predominant and observed on all farms, other commonly observed disease conditions in lactating cattle were pneumonia and foot rot.

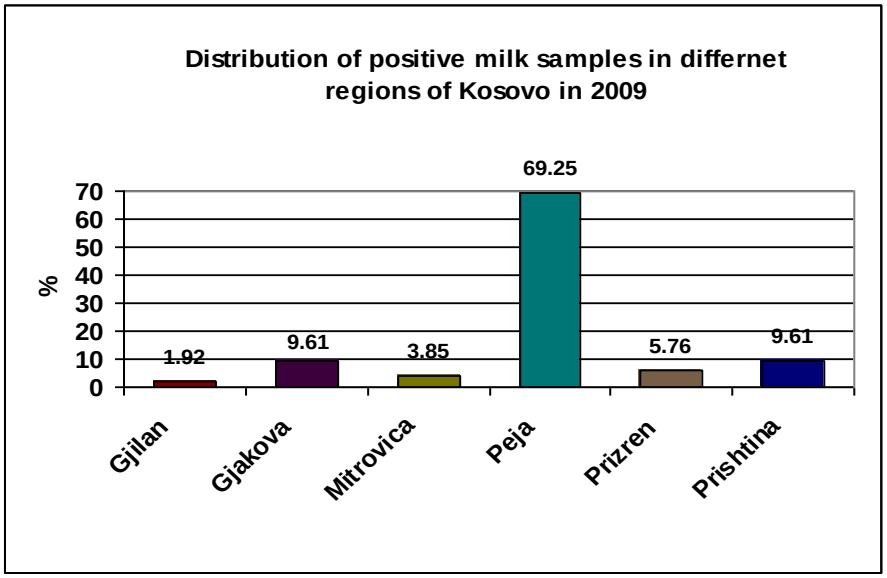
By regions studied milk samples were contaminated with antibiotic and sulphonamide respectively, with the highest incidences in the milk samples collected from Gjakova in 2008 with 36% followed by Peja 24% and with no detected positive samples in Gjilan Region. Distribution of positives is shown in figure 10.

Figure 10 Distribution of positive milk samples in different regions of Kosovo in 2008



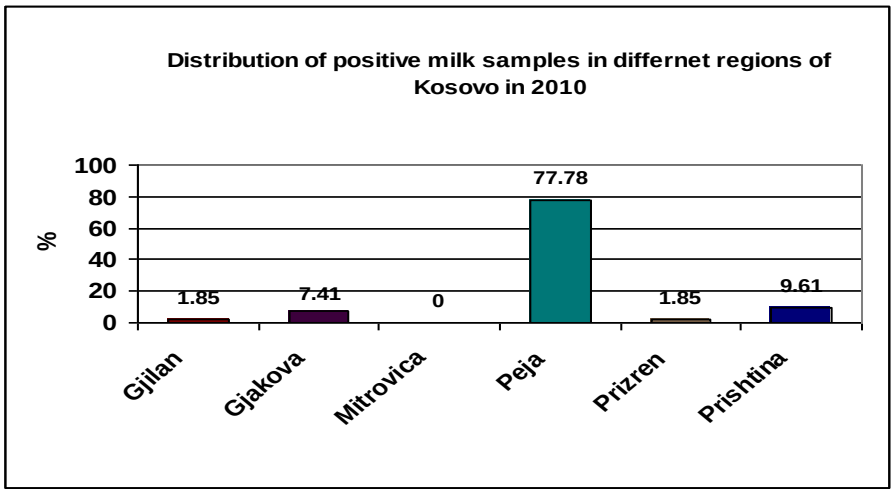
In year 2009 the highest incidences in the milk samples were registered in Peja (69.25%) and compared to that significantly less in other studied regions in Kosovo with lowest findings in Gjilan (1.92%) and Mitrovica (3.85%) as it can be seen in figure 11.

Figure 11 Distribution of positive milk samples in different regions of Kosovo in 2009



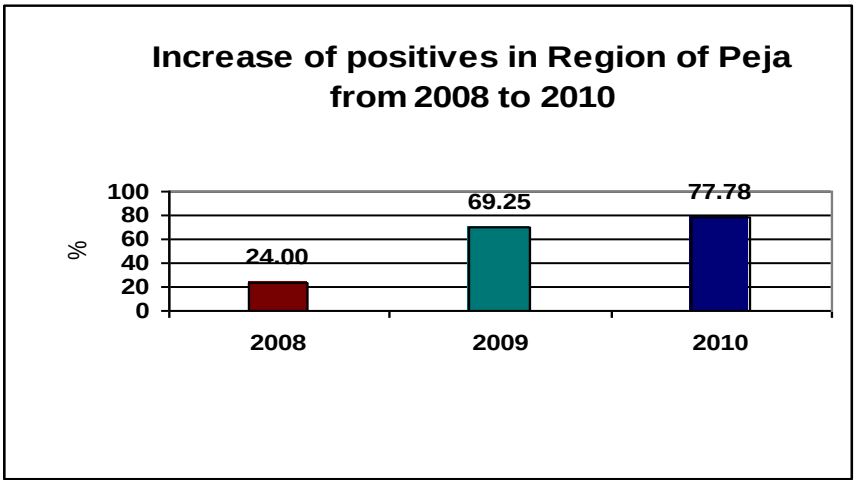
During year 2010, the trends from 2009 continued where Peja Region is found to have highest positive samples number (77.78%) and less in other studied regions with no positive samples detected in Mitrovica Region. Distribution of positives is shown in figure 12.

Figure 12 Distribution of positive milk samples in different regions of Kosovo in 2010



Comparing to other regions significant increase of the incidence in the milk samples has been shown in Peja region; whereas, in 2008 were detected 24% positive samples, in 2009 69.25% and in 2010 reaching the highest level, 77.78%. The increase of positives is shown in figure 13.

Figure 13 Increase of positives in Region of Peja from 2008-2010



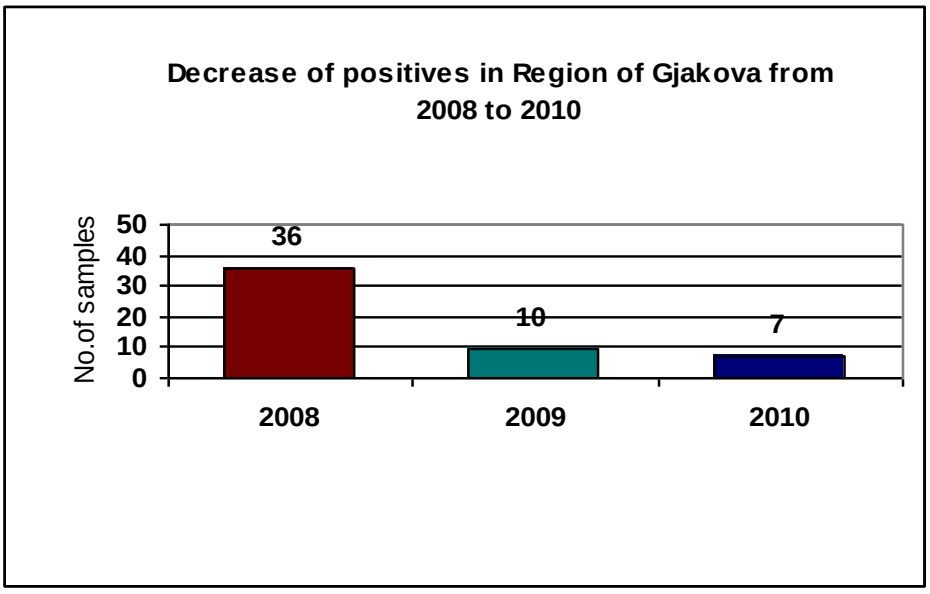
Increase of the positives has been observed also in other regions other than Peja during the period of 2008 to 2010. Thus Prishtina Region in 2008 had 3 positive samples, in 2009, 5 and in 2010, 6 samples, Gjilane region in 2008 was with no positives while in 2009 and 2010 with one sample as can be seen in table 17.

Table 17 Increase of positives in different regions from 2008 to 2010

Raw milk	Total No. of Positives	Peja Region	Gjilan Region	Prishtina Region
2008	25	6(24.00%)	0 (0.0%)	3 (12.00%)
2009	52	36(69.25%)	1 (1.92%)	5 (9.61%)
2010	54	42(77.78%)	1 (1.85%)	6 (11.11%)
Total	**131**	**84(64.12%)**	**2(1.52%)**	**14(10.68%)**

Comparing to other regions significant decrease of the incidence in the milk samples has been shown in Gjakova region whereas in 2008 were detected 36% positive samples, in 2009 9.61% and in 2010 reaching highest level 7.41%, increase of positives is shown in figure 14.

Figure 14 Decrease of positives in region of Gjakova from 2008-2010



The decrease of positive sample number has been observed also in other regions other than Gjakova during the period of 2008 to 2010. Thus Prizren Region in 2008 had 3 positive samples, in 2009, 3 and in 2010, 1 sample, Mitrovica region in 2008 had 4 positives in 2009 only 2 and in 2010 no positive samples. Decrease of positives is shown in table 18.

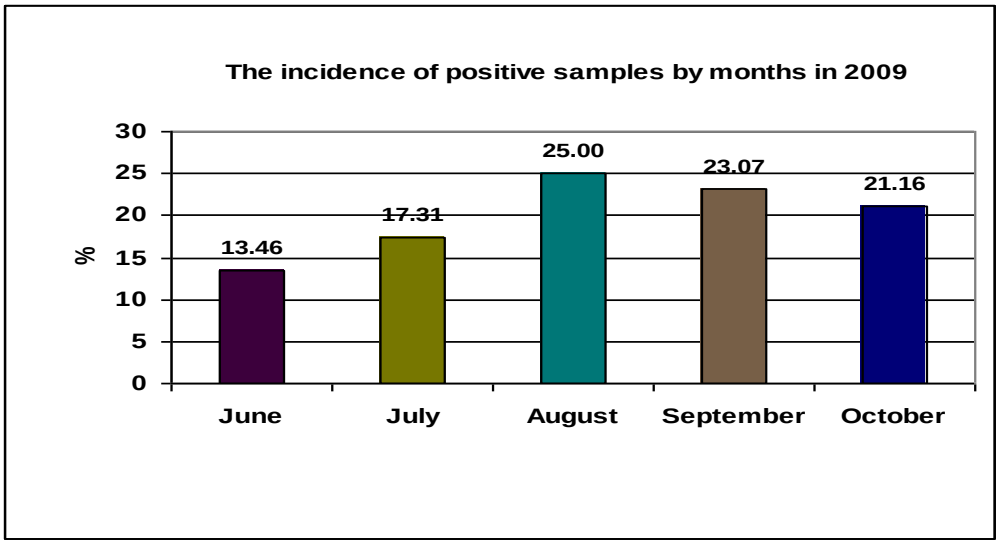
Tab 18. Decrease of positives in different regions from 2008 to 2010

Raw milk	Total No. of Positives	Prizren Region	Gjakove Region	Mitrovica Region
2008	25	3 (12.00%)	9 (36.0%)	4 (16.00%)
2009	52	3 (5.76%)	5 (9.61%)	2 (3.85%)
2010	54	1 (1.85%)	4 (7.41%)	0 (0.00%)
Total	**131**	**7 (5.34%)**	**18 (13.74%)**	**6 (4.58%)**

Antibiotic residue violations seem to occur more randomly through the year, and no seasonal trend could be found. The milk samples with antibiotic residues in year 2008 showed low seasonality in June, August and September and increased in July and October.

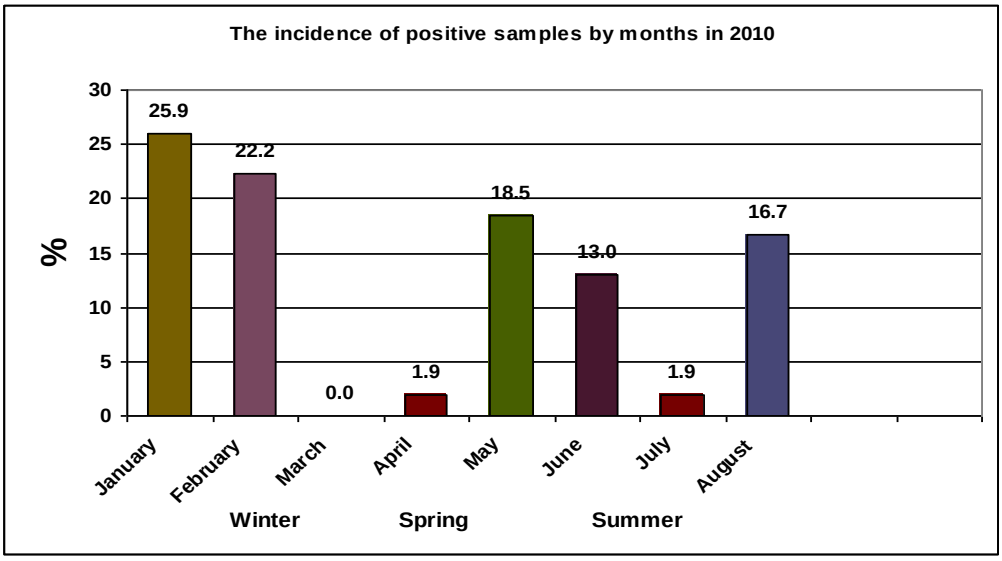
In 2009 no major seasonal trends were found in the incidence of antibiotic residue violations, although the incidence was slightly higher in the summer months, August (25.0%) and September (23.07%). This is shown in figure 15.

Figure 15 The incidence of positive samples by months in 2009



Opposite to 2009 findings in 2010 the incidence was higher in the winter months in January (25.92%) and February (22.22%). The incidence of positives is shown in figure 16.

Figure 16 The incidence of positive samples by months in 2010



The 39 UHT milk samples analyzed were from 8 different commercial brands manufactured in industrial dairy units in Kosovo and from foreign producers. They were collected randomly from retail establishments in the city of Prishtina. According to the methodology used in this study, 38 out of 39 samples were negative,

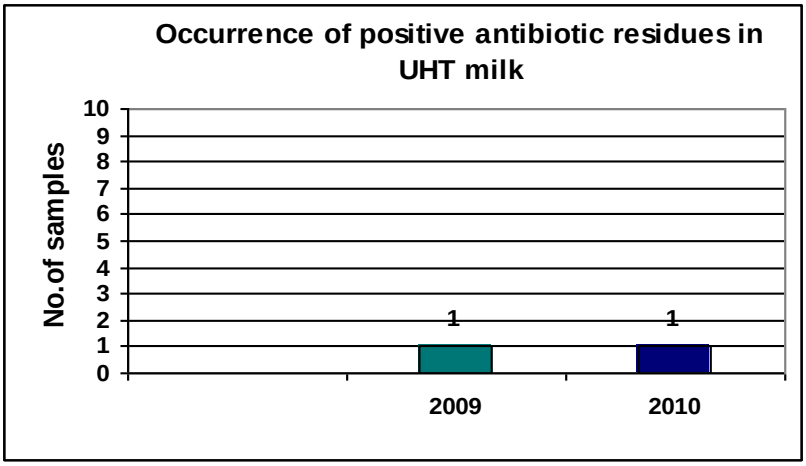
and only one sample was found positive; that sample was from the same commercial brand but coming from the region of Peja, thus reflecting regional farmers diffused 'malpractice', Table 19.

Table 19: Distribution of UHT milk positive samples in Kosovo Provinces

Place	Sample	
	Positive (%)	Negative (%)
Prishtina	0	8
Gjilan	0	8
Gjakova	0	7
Peja	1	7
Prizren	0	8
Total	**1**	**38**

Occurrence of positive antibiotic residues in UHT milk was registered in 2009 with one sample and in 2010 one sample respectively, as may be seen in figure 17.

Figure 17 Occurrence of positive antibiotic residues in UHT milk



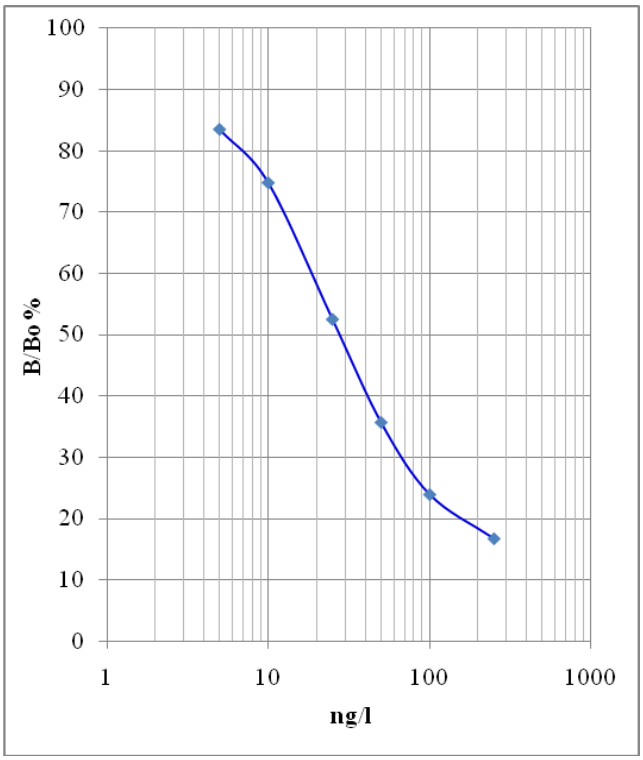
4.2 Results of mycotoxin M1 surveillance in milk produced in kosovo

4.2.1 Validation of the TECNA ELISA KIT MYCOTOXIN M1

To confirm the performances of the Elisa Kit adopted for the aflatoxin M1 determination in milk the laboratory of the KLV participate to the the ring test (*Progetto Trieste 2010, Mycotoxins, Laboratory Proficiency Testing for Food analysis)* that was organized by the producer TECNA and was managed in agreement with the principles of ISO/IEC 170143:2010 and the procedure described in "The international Harmonized Protocol for The Proficiency Testing of (Chemical) Analytical Laboratories", Thompson and Wood(1993). In figure 18 is reported the calibration curve of Tecna ELISA Kit for quantitative determination of Aflatoxin M1 in food. The curve is drawn in a semi-logharitmic graph with a point to point elaboration method. The B/Bo% values on y-axis are calculated dividing the mean absorbance of each standard by the mean absorbance of the zero standards (Bo). On the x-axis is reported the analyte

concentration expressed in ng/L of aflatoxin M1. The concentration of each sample is obtained by interpolation of the B/Bo% value from the concentration curve.

Figure 18 Calibration curve of AFM1.

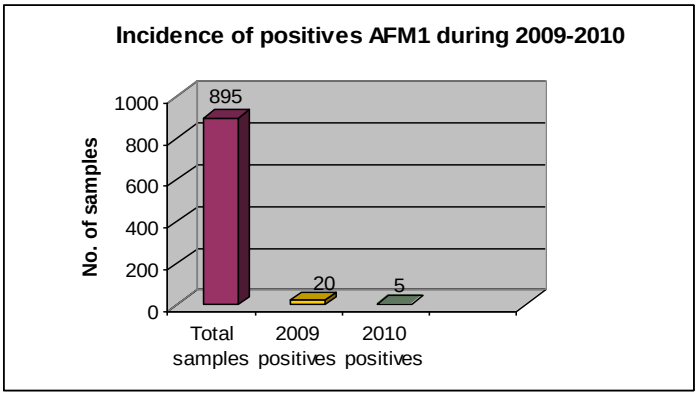


The assigned valued obtained from the ring test called "Progetto Trieste 2010, Mycotoxins" are reported. The laboratory performance evaluation criteria defined z-score = 2 satisfactory 2-3 questionable, >3 unsatisfactory. The results obtained by the laboratory have optimal z-score (0,32 and 0,65 respectively), defined satisfactory from the ring test organizer TECNA srl.

4.2.2 Mycotoxin residues in milk sample collected in Kosovo in the period 2009 -2010

In 2009, 20 samples out of **695** samples(2.88) from MCP were positive, In 2010, 5 samples out of **200** samples examined (2.5%) were positive, At the present study, AFM1 in UHT milk equally with 2 positives in each year were found. The results are shown in figure 19.

Figure 19 Incidence of positives of AFM1 during 2009-2010z



Six hundred and ninety-five milk samples (695) were analyzed in 2009, milk from individual farms (129), milk from collection points (324) and UHT milk (42) collected from different retail markets and brands within Pristine City. While in 2010 the research materials consisted of 200 milk samples, milk from individual farms(60), milk from collection points(110) and UHT milk (30), from retail markets (local and foreign producers), from six different major areas of Kosovo, as it is shown in table 20.

Table 20 Number of positive and negative samples for each kind of milk on AFM1 on 2009-2010

		Collection of milk from different sites during 2009-2010				
		2009		**2010**		
Milk	Total samples	negative samples	positive samples	Total samples	negative samples	positive samples
individual farms	129	129	0	60	60	*0*
collection points	324	506	18	110	107	*3*
UHT milk	42	40	2	30	28	*2*
Total	***695***	*675*	*20*	***200***	*195*	5

In this study 895 milk samples were analyzed by two different screening tests to evaluate the contamination of AFM1 during 2009-2010. Milk samples in 2009 were tested by ELIS-a test (Tecna srl, Trieste, Italy), and 9 samples were tested with SNAP Afla M1 test (IDEXX Laboratories, Westbrook, Maine USA). In 2010 total of 200 milk samples were tested by Enzyme-linked immunosorbent assay (ELISA, Tecna srl,
Trieste Italy) test, and 37 samples were tested with SNAP Afla M1 test(IDEXX Laboratories, Westbrook, Maine USA). The tests used for analysis are summarized in table 21.

Table 21 the tests used for analysis of the presence of Aflatoxin M1 in milk samples.

	Analytical tests used for AFM1 detection during 2009-2010					
	2009			2010		
Analytical Test	No. of tests	No. of contaminated samples	No. of negative samples	No. of tests	No. of contaminated samples	No. of negative samples
ELISA test	695	20	675	200	5	*195*
SNAP AFLA M1	10	0	10	37	0	*37*
Total	*705*	*20*	*685*	*237*	*5*	232

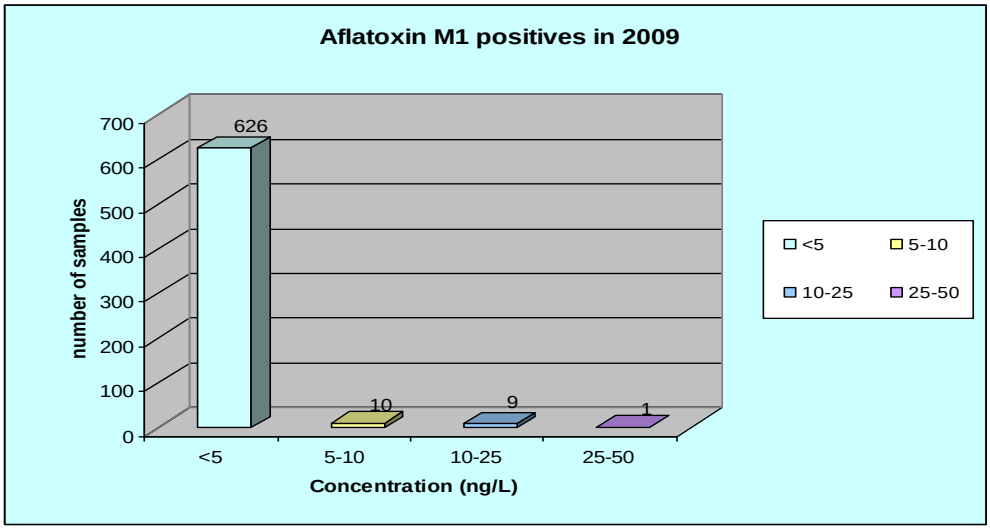
From 895 samples examined, 25 samples were contaminated with AFM1. Thus in 2009, 20 samples out of 695 (2.88%) reacted positively, and 675 (97.12%) were negative, in 2010, of the total 200 samples, 5 samples reacted positive (2.5%), and 195 (97.5%) samples reacted negative; results are given in table 22.

Table 22 Milk samples analyzed to detect the presence of aflatoxin M1 during 2009–2010

Raw milk	No. of samples	No. of positive samples	No. of negative samples
2009	695	20 (2.88%)	675 (97.12%)
2010	200	5 (2.5%)	195 (97.5%)
Total	895	25 (2.79%)	870 (97.21%)

In 2009, 695 samples (97.12%) the AFM1 concentrations were less than 5 ng/L and in 20 samples (2.88%) AFM1 was found between 5 and 50 ng/L. From 695 samples tested with ELIS-test 10 samples were in range between 5-10 ng/L, 9 samples between 10-25 ng/L, and 1 sample between 25-50 ng/L. Samples tested with Snap afla M1 test were negative (these samples were randomly selected from positive samples on antibiotics), as shown in figure 20.

Figure 20 Number of positive and negative samples for each kind of milk on AFM1 during year 2009



In 2010, results yield by ELISA test show that in 195 samples (97.50%) the AFM1 concentrations were less than 5 ng/L and in 5 samples (2.50%) AFM1 was found between 5 and 10 ng/L. All tested samples with SNAP Afla M1 test were negative. The AFM1 level in each sampling region at the indicated concentration of contaminated samples is shown in figure 21.

As depicted in Tables 23, AFM1 was detected in 2% of the UHT milk samples. The AFM1 level in each sampling region at the indicated concentration of contaminated samples is shown in Figure 22.

Figure 21 Number of positive and negative samples for each kind of milk on AFM1 during year 2009

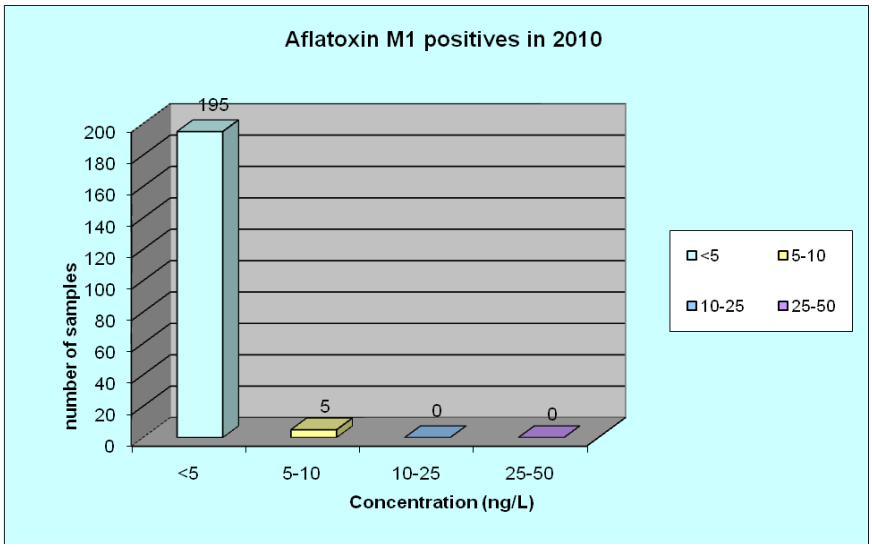


Table 23 Prevalence of contaminated samples with AFM1 in different areas of Kosovo

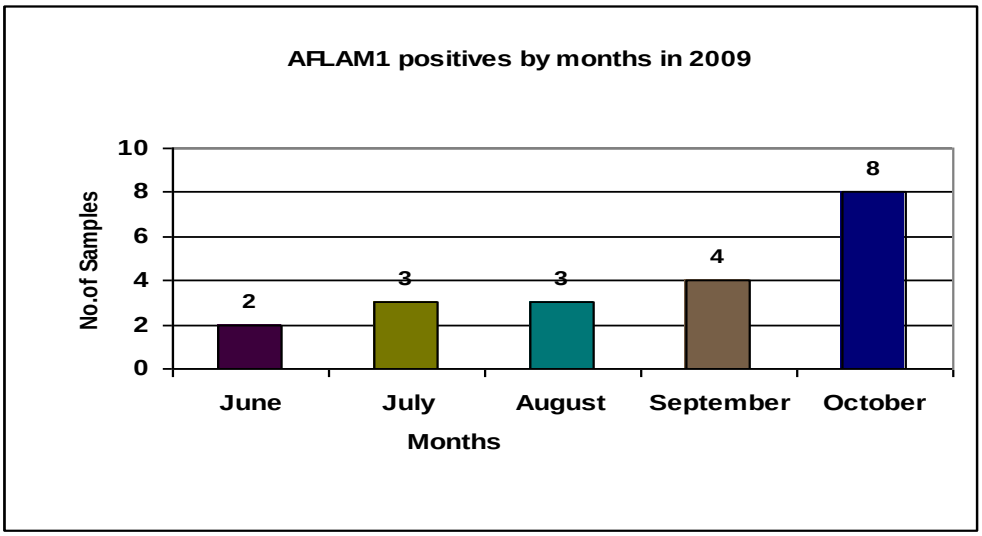
Percentage of contaminated samples during 2009-2010						
	2009			2010		
Location	No. of samples	No. of contaminated samples	Percent %	No. of samples	No. of contaminated samples	Percent %
Gjilan	28	2	7.14	34	0	*0*
Gjakova	29	1	3.44	16	0	
Mitrovica	28	0	0	20	28	
Peja	217	11	5.06	54	3	
Prizren	105	2	1.90	33	0	
Prishtina	175	2	1.14	38	0	
UHT milk	42	2		30	2	
Total	*686*	*20*	*20*	*195*	*5*	5

Two samples positive for M1 were registered during June. Three positive samples each were found in July and August while in September four positive samples were found and in October maximum of positive samples eight (8) were detected. The samples collected in September and October 2009 ranged between

5.23-26.59 ng/L, while the samples obtained in June, July and August 2009 ranged between 5.52=14.82 ng/L AFM1. The highest AFM1 value detected was 26.59 ng/L which was detected on October.

High AFM1 concentrations were obtained from milk samples of September and October, and partly June, July and August had presence of AFM1 concentration, this can be seen in figure 22.

Figure 22 Aflatoxin M1 positive sample and month distribution in 2009.



The results obtained on the distribution by month of the positive, negative, and doubtful samples and on the level of contamination in the positive samples in 2009 are shown in Table 24.

Table 24 Distribution by month of milk samples and aflatoxin M1 concentration in 2009

Mo	No. of samples:			
	Negative	Doubtful	Positive	Contamination level (s) (ng/L)
April	48	48	0	
May	48	48	0	
June	90	88	2	7.92, 7.61
July	90	87	3	7.14, 5.62, 13.56
August	90	87	3	5.76, 5.52, 8.49
September	120	116	4	15.67, 19.11, 14.82, 11.05
October	250	242	8	18.83,20.07,13.82,5.23,16.74,7.92,26.59

The results obtained in 2010 on the distribution by month of the positive, negative, and doubtful samples and on the level of contamination in the positive samples are shown in Table 25. One sample positive for M1 was registered during November, two positive samples were found in December, and with one positive sample each was found in January and February.

The samples collected from November to March 2009 ranged between 6.30-9.81 ng/L, while the samples obtained in February had highest concentration of 9.81 ng/L AFM1, data are summarized in table 25.

Table 25. Month distribution of milk samples and aflatoxin M1 concentration in 2010

	No. of samples:			
	Tested	Negative	Positive	Contamination level (s) (ng/L)
November	40	41	1	6.70
December	50	48	2	8.58,6.30
January	40	39	1	6.72
February	40	39	1	9.81
March	30	30	0	

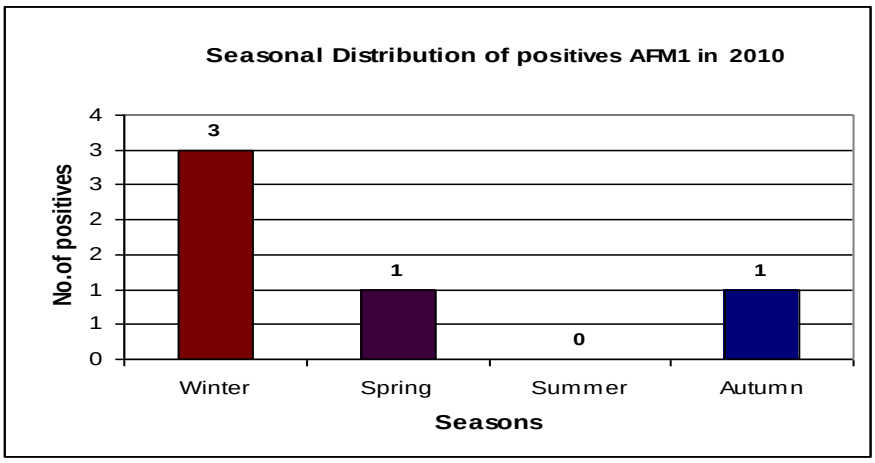
In terms of seasonal distribution, we found the highest number of positive samples in summer (8 samples) and autumn (12 samples), while in winter and spring No positive samples were detected (table 26).

Table 26 . Seasonal distribution of milk samples and aflatoxin Ml concentration in 2009

Seasons	No. of samples:				
	Tested	Negative	Doubtful	Positive	Range (ng/L)
Winter	0	0	0	0	
Spring	96	96	0	0	
Summer	270	262	0	8	5.52-8.49
Autumn	370	358	0	12	5.23-26.59

In 2010 in terms of seasonal distribution, we found the highest number of positive samples in winter (3 samples), spring (1 sample) and in autumn (1 sample), this distribution can be seen in figure 23.

Figure 23 Seasonal Distribution of positives AFM1 in 2010



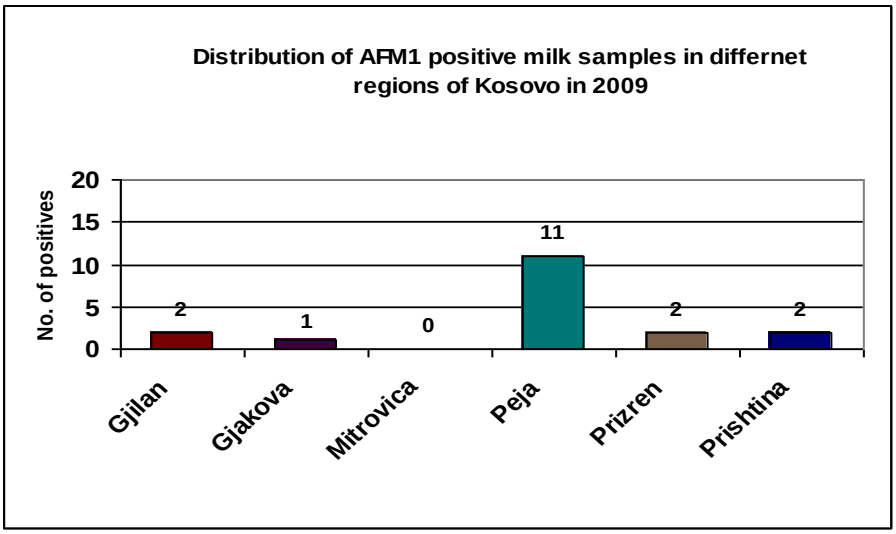
The highest concentration of aflatoxin was registered in one spring sample; it contained 9.81 ng/L. in winter samples ranged between 6.60 and 8.58 ng/L and one sample in autumn containing 6.70 ng/L, this may be seen in Table 27.

Table 27. Seasonal distribution of milk samples and aflatoxin Ml concentration 2010

	No. of samples:				
Seasons	Tested	Negative	Doubtful	Positive	Contamination levels (ng/L)
Winter	130	126	0	3	8.58, 6.30, 6.72
Spring	40	38	0	1	9.81
Summer	0	0	0	0	
Autumn	30	0	0	1	6.70

In 2009, Milk collected from individual farms and Milk Collection Points from different regions of Kosovo, Peja was the region with most of positive samples 11 or (61.11%) while Mitrovica region was without contaminated samples. Data obtained for distribution of positives during 2009 are summarized in figure 24.

Figure 24 Distribution of AFM1 positive milk samples in different regions of Kosovo in 2009



In 2010, Peja was the region with 100% positives, no contamination in other regions of Kosovo, as it is shown in figure 25

Figure 25 Distribution of AFM1 positive milk samples in different regions of Kosovo in 2010.

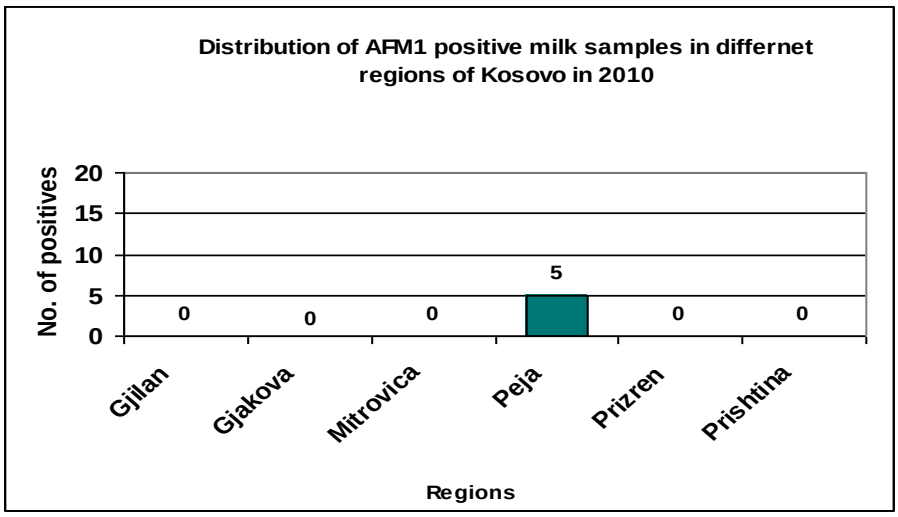


Figure 26 Aflatoxin M1 contamination by months in 2009

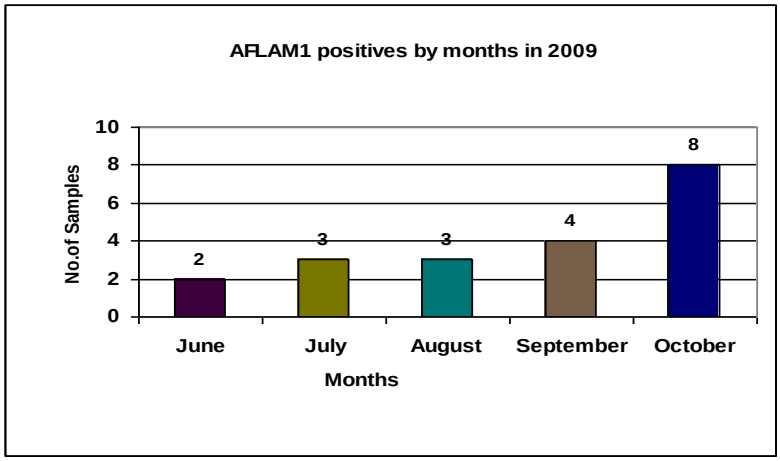


Figure 27 Distribution of positive milk samples in different regions of Kosovo in 2009

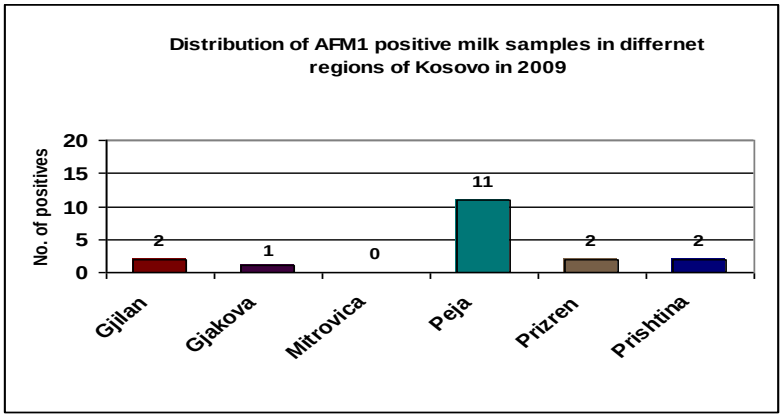


Figure 28 Seasonal distribution of positives AFM1 in 2009

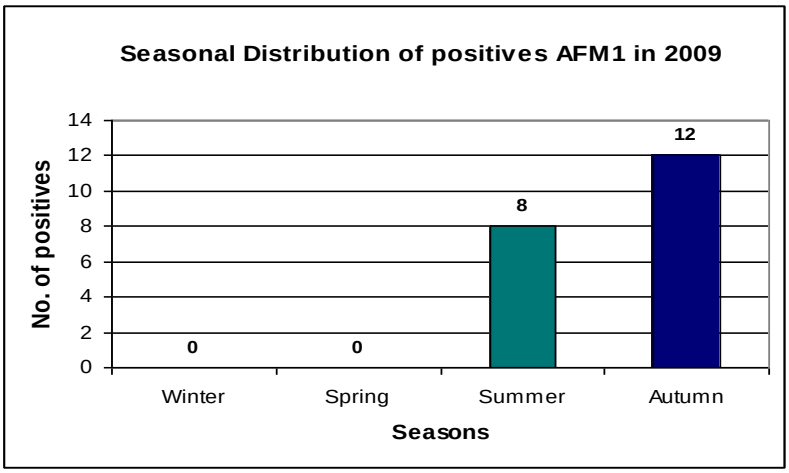
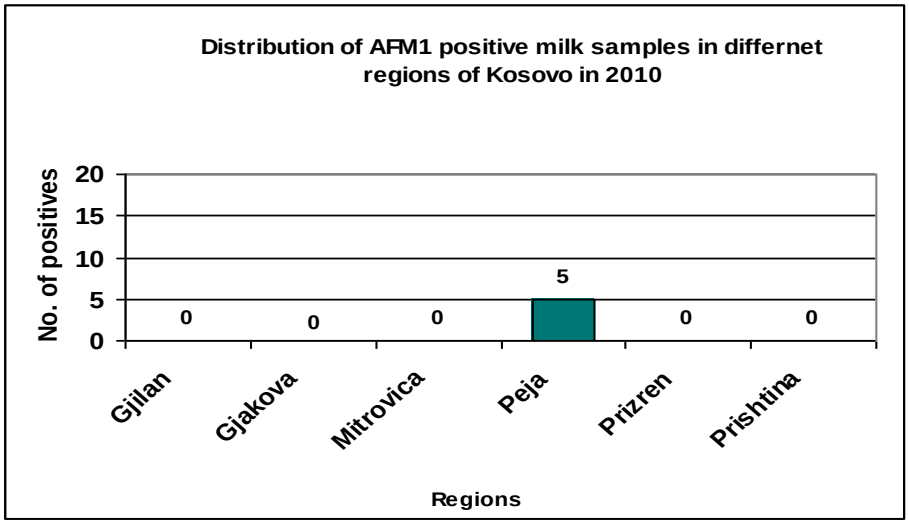


Figure 29 Distribution of AFM1 positive milk samples in different regions in 2010



5. DISCUSSION

5.1 Residue of antibacterials in milk

In the present study, the analyses showed that milk samples collected from different parts of Kosovo were contaminated with different groups of antibacterials and among them, ß-lactam residues were the major contaminants. In order of frequency, the active ingredients most commonly used were penicilins, tetracyclines, and sulphonamides, making up over 90 % of all antimicrobials used for disease treatment. In farms antibiotics used for the treatment of cattle were administered mainly by veterinarians (64.70% of cases), owner/manager, other than the veterinarian, accounts for the 35.30% of the cases this findings were in agreement with data by Kaneene *et al.*(1992). The results of the sampling campaign 2009 gave an overview of milk contamination by veterinary drugs more complete than those obtained in 2008. The results were in agreement with findings from other developing countries like Brazil as observed by Carolina *et al.* (2009). In that study from the total of 103 milk samples antimicrobial residues were detected in 11 (10,68%) samples, and Folly *et al.* (2001) reported that among the 300 milk samples collected in Rio de Janeiro region, 13 were positive, revealing a frequency of contamination 4.33%.

Other studies carried out in Kenya (Shitandi, 2001), 21% of 1109 milk samples were positive for antibiotic residues, while a survey in Trinindad by Adesiyun *et al.* (1997), showed that 10.8% of milk samples were positive. In Turkey, in a study by Ceyhan & Bozkurt (1987), from a total 200 milk samples collected from Ankara region, the 5.5% was positive for antibiotic residues, whereas another antimicrobials survey in milk by Aydmn *et al.* (1989), reported that the 44% of 204 raw milk samples, was positive for antibiotic residues. Gacnik *et al.* (2000), studied residues of antibiotics in the four year period (1995-1998) in Slovenia and a total of 3358 milk samples were analyzed; the majority of them (99.4%) were negative and antibiotics were found in only 19 samples (0.60%). In Northern Germany, Suhren, *et al.* (1994) found 2.8% of the total milk samples (2972) investigated were positive by the Delvotest SP test control.

Data reported by several researchers as those recorded in Turkey (Oruc, 2005), Kenya (Ombui, 1994) and Kuwait (Al-zenki, 2007), studying antibiotics in milk for human consumption and the reports of the annual surveillance for veterinary residues in food Slovenia (Dolajs, 2007) and in the UK (2007) collected showed that no residues were detected at concentrations at or above the relevant Reference Points in milk. Observations reported by several researchers (Allison, 1985; Booth & Harding, 1986; McEwen *et al.*, 1991; Mitchell *et al.*, 1998;Ghidini *et al.*,2002; Holstage *et al.*, 2002; Ramírez, *et al.*, 2001; Allara *et al.*, 2002; Khaskheli *et al.*, 2008) confirmed as in our study penicillins were the main group detected and these findings reflected the frequent use of intra-mammary infusions containing beta-lactams, as we can suppose occur in Kosovo for the treatment of mastitis of milking cows.

Tetracycline content in our positive samples was above MRL of 100 μg/kg set by EU and it corresponds with findings reported by Jacques *et al.* (1998) who studied the detection and identification of tetracycline residues in 973 samples of bulk milk randomly collected by milk producers in the Netherlands.

Medeiros *et al.* (2004), who used enzyme immunoassays IDEXX for the analysis of 30 samples and it was observed that 43.33 % of the total samples had residues of antibiotics with tetracycline as the most frequent.

Contradicting the literature where penicillin is the antibiotic most frequently found in the milk market residues of beta-lactam were not found in the studies by Navrátilová (2009), in Czech Republic In the 170 milk samples checked only low concentrations of tetracycline antibiotics residues were detected, though all the analyzed samples revealed residues of tetracycline. Only oxytetracycline residues were detected in 50.6% of analyzed samples, also in the study reported by Chung in 2009. The total of 1080 samples of fresh milk in Taiwan were analysed and penicillin, tetracycline, were recorded in the 17.5%, of positive fresh milk samples and residues found were mostly below 500 μg/kg in fresh milk One thousand samples of locally produced or imported milk and dairy product samples in Kuwait were analysed and 29.1% of the analyzed local fresh milk samples were above the maximum residue level (MRL) for tested residues with tetracycline as the predominant residue (Alomirah, 2007). In the study by Ben-Mahdi *et al*. (2009) in Algeria, 760 samples of milk were collected and the results showed a 9.87% contamination the residues of penicillin and tetracycline were the main source of contamination of milk samples positive. The prevalence of antibiotic residues in dairy household milk samples by Ekuttan *et al.* (2007) was 4% also in that case with beta lactams and tetracyclines. A survey in Hyderabad (India), by Sudershan RV *et al.* (1995), of 205 milk samples analyzed, 9% were from market samples and 73% from individual animal. All milk samples contained oxytetracycline residues and concentrations ranged from 200 to 6700 μg/kg. In a study by Bando *et al.* (2009), of the State of Paraná, Brazil, 41 of 151 samples contained tetracyclines; shown traces of tetracylcines below MRL In our studies Sulphonamides were found below MRL set by EU and corresponds with findings from other studies, Tolentino *et al.* (2005), who analysed Mexican pasteurized milk during one year (96 samples). Percentage of positive samples to sulfonamide residues were around 50% with sulfonamide residues ranging between 1.9 and 180 μg/kg. Suhren G (1994) in examined the incidence of antimicrobials in car tanker milk in Northern Germany: results showed a total of 2.8% of the samples (n 2972) to be inhibitor positive by the Delvotest SP test; 1.1% as sulphonamides. In a study in Slovenia by Gacnik *et al.* (2000), in the last four year period (1995-1998) a total of 406 milk samples were tested for sulphonamide residues, within the context of the official Slovenian monitoring program and no sulphonamides were found in any tested samples. Though different analytical approaches were adopted, the results found in UHT milk reported in the present study revealed levels higher than those frequently found in other countries. Wit *et al.* (1996), recording the presence of antibiotics in UHT milk produced in Holland stated that 100% of the samples checked were below the EU MRL adopted for the commodity; Bayenes *et al.* (1999), in a similar research in Costa Rica found the total absence of residues.

Kosovo results were in agreement with those reported by Fonseca *et al.* (2008), Movassagh *et al.* (2011), showed that around 5% of cow raw milk was positive for antibiotics residues, and Unusan (2009) found streptomycin and tetracycline in UHT milk samples, even the concentrations found were below the maximum residue limits permitted by the European Union. There was a high incidence rate of tetracycline, with 40 milk samples (66.8%) being contaminated, respectively.

5.2 Residues of AFM1 in milk

Several studies around the world have addressed the issue of AFM1 contamination of milk and milk products. To put our results in perspective, it would be helpful to compare them to those of the sort reported elsewhere. All milk samples analyzed showed AFM1 concentrations lower (highest value 26.2 ng/L). Than the tolerance level of 50 ng/L that is the level accepted by European Union and *Codex Alimentarius* Commission According to the results obtained in Kosovo and other countries, the magnitude of contamination of AFM1 in Kosovar samples was comparable to European countries like Italy, Portugal and Greece (Galvano *et al.*, 1998; Markaki & Melissari, 1997; Martin & Martin, 2000). The concentration of AFM1 in raw milk can be considered very low in Kosovo; indeed the contamination levels measured are lower than the maximum limit fixed by European Union Commission Regulation, even for infant consumption (Commission Regulation, 2006). In the recent past, it has been reported that many countries of Europe showed relatively low levels of contamination of AFM1 in milk and milk products (Trucksess, 1997, 1999). In European countries the occurrence of AFM1 at low levels may be a result of stringent regulation of AFB1 in complementary feedstuffs for dairy cattle. Our study confirmed the low incidence of AFM1 in milk produced in Kosovo as reported in a Greek study with levels of AFM1 in milk by far below the tolerance level (Kaniou-Grigoriadou *et al.*, 2005). Roussi *et al.* (2002) analysed 114 samples of raw and market milk in Greece and only 3 samples (2.6%) were contaminated with AFM1 > 50 ng/L. Grigoriadou *et al.* (2005) reported that in Thesaloniki Province levels of AFM1 in milk were far below the tolerance level (highest value 18.2 ng/L).All results are in agreement with European surveys (Breitholtz-Emanuelsson *et al.*, 1993; Valenta & Goll, 1996; Galvano *et al.*, 1998; Skaug, 1999; Martins & Martins, 2000; Rodriguez Velasco *et al.*, 2003) and also with more recent survey reported for Argentina with not contaminated milk samples detected (Lopez *et al.*, 2003). In Italy, of 161 samples of dairy products, only 4 (2.5%) were contaminated at a level >50 ng/L (Galvano *et al.*, 2001); the other 40 milk samples recorded AFM1 at levels ranging from 4 to 23 ng/L. None of the contaminated samples exceeded the legal limit of 50 ng/L set down by the European Union for milk (Finolic & Vecchio, 2003). The 90 milk samples analyzed in Serbia in 2009 revealed AFM1 in 23 samples of milk from small individual farms exceeding the limits allowed by European Union, all the other commercial milk samples (pasteurized milk and UHT milk) amount of aflatoxin M1, were found but at concentration that does not exceed EU legislation, or legislation which is used in Serbia (Horvatic *et al*., 2009).

Among the 120 milk samples analyzed in Albania in 2001, the 16% was positive for AFM1; the 13% were collected during the winter season and resulted above the 500 ng/L level, while the 3% represented by summer samples exceeded that level (Panariti E, 2001). The 98.4% of milk samples collected in Croatia in 2011 showed levels of AFM1 below maximum tolerance level accepted by the European Union despite the concentrations of few samples AFM1 recorded in winter–spring season were in the range of 35.8–58.6 ng/L while in summer–autumn were in the range of 11.6–14.9 ng/L (Bilandzic *et al*., 2011).

For raw milk samples, the levels of AFM1 contamination reported in the present study where lower than those reported in Turkey where 47% of the analyzed samples (129 sample) contained AFM1 at levels

exceeding the EU accepted limit (Unusan, 2006). High level of contamination were found in Turkey in 2005 as reported by Celik *et al.*, in 85 pasteurized milk samples from several markets in Ankara with the ELISA technique. Seventy-five samples (88.23%) were found to be contaminated with AFM1, and 48 samples (64%) exceeded the legal level of AFM1 in milk according the *Codex Alimentarius*, 50 ng/L (Commission Regulation, 2006). A study on randomly selected samples of raw cow milk from North African countries showed a high level of contamination with AFM1 ranging between 30 and 3130 ng/L (Elgerbi *et al.*, 2004). In Korea, for example, AFM1 was detected in 79% (143 samples) of 180 samples with a mean concentration of 57 ng/L when determined by ELISA. By HPLC, 105 samples (58%) were contaminated with a mean concentration of 71 ng/L (Kim *et al.*, 2000). In a study conducted in India the incidence of contamination of AFM1 in infant milk, milk based cereal weaning food and liquid milk samples was almost in the magnitude of 87% (Rastogi *et al.*, 2004), with 99% of contaminated samples exceeding the EU/Codex limits. Occurrence of AFM1 in raw milk samples from 14 districts of the Punjab Province, Pakistan has been reported by Hussain & Anwar (2008). It was found that of all samples analysed were contaminated with aflatoxin M1 (AFM1) and the 99.4% samples exceeded the EU limit. Occurrence of AFM1 in milk from two different provinces in Iran has been observed. Kamkar (2005) reported that in 85 out of 111 samples from Sarab Province, AFM1 was detected at concentrations ranging between 15 ng/L and 280 ng/L and in the 40% of positive samples was higher than the maximum tolerated limit. In Shiraz province, AFM1 was found in 100% of the 640 milk samples examined and the 17.8% of samples had an AFM1 concentration greater than the maximum tolerated limit (Alborzi *et al.*, 2006). Tajkarimi *et al.* (2007) reported the contamination of AFM1 in 98 samples of raw milk from milk tanks in one dairy plant in each of five regions in Iran. In Khorasan province, 196 samples were collected and AFM_1 was found in all the examined milk samples with an average concentration of 77.92 ng/L. 80.6% of the samples had AFM_1 greater than the maximum tolerance limit (Sani *et al*., 2010) .

Fifty four samples of pasteurized milk produced by five different dairies from Morocco were surveyed for the presence of AFM1 and 88.8% of the samples were contaminated with AFM1; 7.4% being above the maximum level of 50 ng/L set by the Moroccan and European regulations for AFM1 in milk (Zinedine *et al.*, 2007).

At the present study, AFM1 levels were determined in ultrahigh treated temperature (UHT) milk which is mostly consumed in the big cities of Kosovo. AFM1 was detected in 2% of the UHT milk samples. These results are in parallel with the findings of several reports (Aycicek *et al.*, 2005; Gurbay *et al.*, 2006; Sarımehmetoglu *et al.*, 2004; Unusan, 2006) which pointed out the presence of AFM1 in more than 60% of the UHT milk.

However, a number of researchers (Blanco *et al.*, 1988; Gurbay *et al.*, 2006; Gurbuz *et al.*, 1999; Raza, 2006; Yaroglu *et al.*, 2005) reported a lower incidence for AFM1 in UHT milk.

It has been indicated that many countries in Europe showed relatively low levels of contamination of AFM1 in milk samples because of a result of stringent regulation of AFB1 in dairy cattle feed (Trucksess, 1999, 2006). A total of 107 samples of raw, pasteurized and UHT milk commercialized were analysed and 79 milk

samples, were positive with concentration ranging from <0.02 to 0.26 ng/L; the 6.5% was contaminated more than 50 ng/L (Shundo & Sabino, 2006).

It was also observed in our studies we found that during the autumn and winter months, contamination levels in the samples were higher than they were during the summer months. This could be explained by the prolonged storage required for feed, which would provide favorable conditions for fungi growth or the use of contaminated feed for the animals in autumn and winter months, The effective factor on low AFM1 level in June, July and August was most likely out-pasturing of milking animals. This similar result was stated by some other researchers (Blanc & Karleskind, 1981; Applebaum *et al.*, 1982) and they found that low AFM1 level production was obtained in summer season. Therefore, it is possible to say that the results obtained by us were parallel to the results of prior studies. Even though there was significant difference in the AFM1 levels among regions, the AFM1 level in Peja region tended to be higher than that in the regions of Kosovo The seasonal samples were taken equally from all of the studied regions. However, it was only proven that the values for the Mitrovica region were significantly lower than those for other regions. Positive samples n 2009 were found only in summer and autumn while in 2010 positive samples were found in the winter and spring. In our studies higher concentration of AFM1 were found in cold seasons as compared to hot seasons, however our investigation indicated that the value of AFM1 concentration in summer of 2009 was higher than in summer of 2010, which suggests that other factors have a major influence on the AFM1 level in milk, at least in Kosovo. Seasonal effect influences concentration of aflatoxin M1 as it is shown in figure 28. This result seems to be consistent with the report of Blanco *et al.*, 1988; Lopez *et al.*, 2003; Rossiet *et al.*, 1996; Tajkarimi *et al.*, 2007; Kamkar, 2006) who reported a higher incidence of AFM1 contamination during cold seasons than hot ones. Another peculiar aspect of this study was that milk samples were collected from different regions with different mean relative humidity and day/night temperature variation, that could exert significant influences on fungi growth and aflatoxin production. In Kosovo, for instance, the temperature and relative humidity is higher in Peja Region than the other regions (Zajmi, *et al.*, 2002).

This humid climate in Peja region might give rise more easily to the molding of feeds for dairy cattle and consequently to contamination of milk with aflatoxins. Studies by Jay (1992) shows that some moulds like *Aspergillus flavus and parasiticus* can easily grow in feeds having substrate moisture between 13% and 18% and environmental moisture between 50% and 60%. Furthermore, they can produce toxin under conditions of 25°C and 85% and 90% relative humidity.

6. CONCLUSIONS

The present investigation is the first performed in Kosovo to evaluate the presence of antibiotic residues in foodstuffs, especially in milk and milk products. The results show that antibiotic residues can be found in milk produced for human consumption in our country and suggest unsatisfactory quality of milking practice and of UHT production techniques with respect to the control of such parameter.

There were a significant number of positive samples of antibiotic residues in milk for human consumption from different collection sites in Kosovo: the highest number of positive samples were found in 2008 in the Gjakova region followed by Peja < Mitrovica < Prizren < Prishtina < Gjilan. In 2009 in Peja region had the highest number, followed by Gjakova <Prishtina <Prizren <Mitrovica <Gjilan. In 2010, the highest number of positive samples were found in Peja region followed by Prishtina <Gjakova Prizren <Mitrovica <Gjilan.

Increase of positive samples during 2008-2010 were registered in Peja Region, whereas decreases of positive samples were observed in Gjakova Region. Antibiotic residue violations seem to occur more randomly through the year, and no seasonal trend could be found.

The incidence of AFLA M1 contamination in raw milk samples was present mostly in the northwest of Kosovo in the Peja Region, which is possibly because of higher temperature and moisture in comparison with other regions of Kosovo. The results of this survey indicated very low levels of Aflatoxin M1 in raw milk and no one of the contaminated samples exceeded the EU limits.

The AFLA M1 levels showed a seasonal variation. Our experiment confirmed this influence, since the highest numbers of positive samples were found in autumn winter and spring.

The most frequent presence of contaminants has been found in Peja Region. In the future, there is a need for further research in Peja Region to determine the causes and consequences of frequent presence of antibiotics in milk.

Given the considerable levels of antibacterials residues detected in raw milk, the worrying consequences for human health prompt a number of recommendations that should be addressed to public authorities, veterinarians, livestock producers and consumers.

Competent authorities should apply continuous monitoring programs to obtain safe milk products offering no health risk to Kosovo consumers. Further to that is mandatory a program of continuous education in veterinary medicine to inform veterinary practitioners on the prudent and safe use of antibiotics that should be used in animals respecting authorized dosages, route of administration, length of therapy and withdrawal times. The lack of an authority for national registration of veterinary products and the use of imported medicinal products or the adoption of black market preparations can be one of the reason for unattended withdrawal time after treatment and for the presence of antibiotic residue at level above the permitted MRL.

In addition, more research should be carried out to accurately focus the problem and implement effective corrective actions, to reduce milk contaminants. In this perspective, the Food control laboratories should choose, use and evaluate screening tests for milk residue control, and such tests should of easy use, suitable for a broad spectrum of antimicrobials and, considering the economic resources of a country like Kosovo, as inexpensive as possible, to reduce at least the monitoring costs.

The results of the survey on aflatoxin residue indicated very low levels of aflatoxin M1 in raw milk and no one of contaminated samples exceeded the EU limits. The results suggest that Kosovar fresh milk samples can be considered safe for human consumption. The survey was a first step to assure consumer protection against the risk of aflatoxin exposition, and our data might give preliminary indications about the contamination profile with aflatoxin M1 but since there is not other study done in Kosovo about the aflatoxin M1 content of milk and dairy products, more studies are required in the near future.

Considering the reported results, it could be concluded that aflatoxin M1 incidence in samples selected from marketed milk in Kosovo, at the moment, does not appear to be a serious public health problem.

The results of the study indicate that the incidence of AM1 contamination in raw milk samples was present mostly in north west of Kosovo in Peja Region which is possibly because of high sorghum silage and grain production in this area, which could be among the common sources of AFB1 contamination, and also higher temperature and moisture in comparison with the other regions of Kosovo.

Results of this study indicated that the AFM1 levels had seasonal variations, since the highest numbers of positive samples were found in spring and autumn. These data likely reflect the fact that during autumn and winter, animals are fed mixed feeds, the most common vehicle for aflatoxin B1.

As this is the first study on aflatoxin M1 occurrence in Kosovo, the continuous monitoring AFM1 concentration in milk should alarm us as the possible concentration in milk products could increase the contamination levels. There is an urgent need to create awareness among milk producer and veterinary practitone and farmer about the health hazard caused by the contamination of milk with AFM1.

Therefore, it is important to inform producers and consumers about the toxicity potential of aflatoxins in order to reduce their potential health risk and economic loss. In this regard, organization of official training programs should be considered by the government aimed for producers, which should work with principles of good manufacturing and good storage practices, and also, stringent quality control during processing and distribution of these products. Thus the priority for national authorities is to start information campaign for farmer and milk producers to ensure the quality of feed by controlling the time of harvest the storing condition by monitoring temperature and humidity of feed and protecting stored feed by contamination.

Frequent analytical surveillance by food control Agencies is highly recommended to control the incidence of mycotoxin contamination in Kosovo especially in animal feed and dairy products. KFVA must be start developing much needed Quality Management Systems, the laboratories to learn how to document the quality of existing analysis, and for the laboratories to learn new methods. In order to obtain reliable results, and hence give consumers and producers confidence in testing methods, there is an urgent need for internationally validated methods, which could serve as confirmatory methods and form the other main pillar in a reliable and cost effective measurement and prevention strategy.

The development of new and improved analytical methods, verified by proficiency testing programs that meet internationally accepted standards, should be the main scope of our national reference laboratories.

The thesis represented the first moment for training laboratory personnel on new equipment, with new methods; It was a starting point to increase the laboratory capabilities, efficiency and competence.

During the work done for the thesis the KFVA laboratory participated with a good results to the ring test organized by the producer for aflatoxin screening kit. This activity should be continued and increased as it offers opportunities to exchange scientific knowledge and improve the researcher'confidence with analytical techniques.

Together with the implementation of appropriate legislation, adequate and controlled sampling network, we will be able to provide effective mechanisms for food control, appropriate risk-assessment and consequent reassurance for the consumer.

Because it is perceived as a food safety issue, the topic of contaminant residues in food is a highly emotive one and can elicit a strong public reaction that can adversely influence both domestic and international markets. Strengthen consumer confidence, through the industry's ability to promptly identify and recall potentially unsafe product, by providing reliable information business to business, to consumers, to government inspectors, to financial or technical auditors.

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