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Research Article

DETECTION AND RISK ASSESSMENT OF BETA-LACTAM RESIDUES IN KOSOVO'S MILK USING ELISA METHOD

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ABSTRACT

Objective: Beta-lactams are used most commonly in the treatment of gram positive and gram negative bacterial infections in people and animals. However, their extensive use often not following any safety criteria, in many cases, has led to the contamination of milk intended for human consumption, which can have serious and unanticipated implications for human health. The aim of this study is to detect and assess beta-lactam residues in Kosovo's milk, and to evaluate the exact application of antibiotics on Kosovo's dairy cattle.

Methods: 127 samples of raw milk were collected from 80% of dairy farms in Kosovo that were visited during 2011. The samples were analyzed in the laboratories of the Veterinary Institute in Skopje using Elisa screening method. The drug use and mode of delivery was assessed through a specific questionnaire which identifies problems with drug treatment of cattle.

Results: From 127 milk samples, 64 of them or 50.4% were contaminated with beta-lactam residues, while 15or 11.6% already passing the maximum residue limit. Cows treated with beta-lactams78or60% of the total received the drug intramuscularly and 40 or 31% of total received it intramammary.

Conclusion: We conclude that there is a clear risk involved in the consumption of dairy milk, as far as the presence of antibiotics is concerned. Therefore, it is vital that all inspection controls are strengthened to monitor records on the treatment of animals as well as the proper use of antibiotics.

Keywords: Antibiotics, Beta-lactams, Dairycattle, Residues, Milk, Elisa.

INTRODUCTION

Beta-lactams are the oldest and most widely used antibiotics for the treatment of gram positive and gram negative bacterial infections in man and animals. They have a broad spectrum of bactericidal action killing bacteria by inhibiting bacterial cell wall synthesis.

These antibiotics, the main being, penicillins, cephalosporins, carbapenems and monobactams in their corecontain the β-lactam ring (Fig.1), which is a solid structure with side groups that can modify the molecular biochemistry in their molecular structures[1].

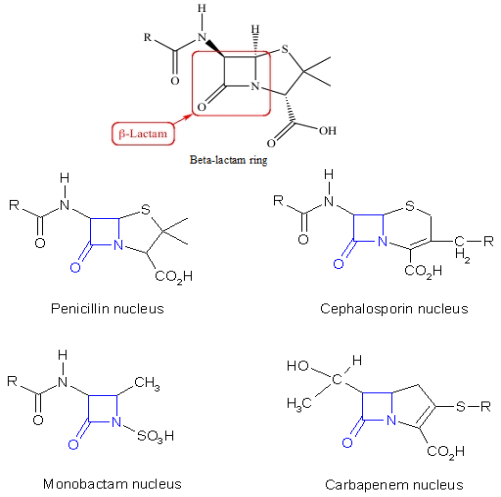

Fig. 1: It Shows the beta-lactam core structure

Beta-lactams have the same mechanism of action in the organisem of dairy cattle as in that of humans. They act inhibiting linkages of proteins to them, which normally, catalizes interconnection of bacterial cell walls. So the bacteria which in the presence of penicillins cannot be separated, respectively, multiplied, are destroyed dropping their cellular walls in the process[2].

Bactericidal action of beta-lactam antibiotics is directly affected by their ability to react with penicillin binding proteins (PBP)[3]. Beta-lactams ring is an entity very much intense and chemically highly reactive. Reaction between Peptidoglycan and Beta-lactams results in an irreversible inhibition of PBP and the destruction of bacterial cell. For instance, when proteins bind with penicillins they deactivate and as such are unable to play part in the synthesis of the cell wall, which in turn, enables the penicillins a much easier access into the cell walls, of both gram positive and gram negative bacteria[4].

Each newly given antibiotic, in any way whatsoever, except the ones given intrammarly, before becoming residues in milk pass through long and complex physical and metabolic processes in the organisem of cattle, from absorption to their excretion, which can be seen in the figure (Fig.2) [5].

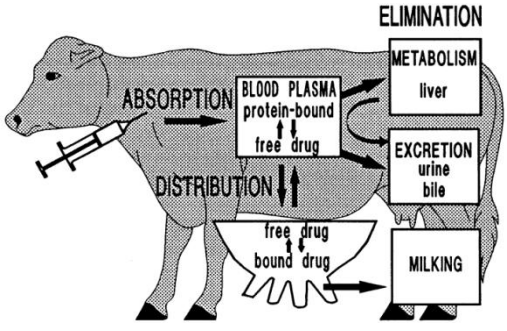

Fig. 2: It Shows the pathway model: Blood is central to the distribution of drug through tissues and metabolic and excretory organs (Reybroeck 2010).

Here, too, the drug quantity and its metabolite concentration during circulation in tissues and body fluids, must be taken into account [6].

Beta-lactams dissolve very quickly and through blood circulation are easily distributed to tissues, organs and body fluids, including the cerebrospinal fluid. Their maximum concentration can be observed

after 2-3 hours, while their action could take from 6-12 hours. These antibiotics are metabolized mainly in the liver and then are excreted through glomerular filtration [7].

Milk is one of the main foods that plays a crucial role in human health and life. It is a liquid substance full of nutrients[8].

It must be noted that antibiotic residues in milk and other food products of animal origin are a major concern to public health and the dairy industry[9]. Allergic reactions caused to the consumer are highly diagnosed, especially at children. The reactions occur most often on skin and are anaphylactic, which may cause even death[10].

Other risk factor is the emergence of betalactam multi-resistant bacteria, which poses a major concern for the world wide population, since 6 to 8% of the global population are sensitive to them [11]. The resistance is usually stimulated by cellular mechanisms during which bacteria produces enzymes that share two beta-lactam rings, known as beta-lactamase[12].

Besides being a major health concern, antibioresistance as a factor, is also a great economic burden to the world society. To combat it billions of dollars are being spent on the production and introduction of new drugs into the market[13]. However, the extensive and agressive comercialization of newer drugs has also led to the enourmous abuse in their production, illegal marketing and smuggling.

During the treatment of diseases with antibiotics veterinarian's recommendations should be respected regarding the application method an duration of their administering[14].

The most frequent diseases diagnosed in dairy farms in Kosovo are: mastitis, lacticacidosis, dermatofibroma, ketosis, gastroenteritis, viral diarrhea, bronchopneumonia, chronic stomach tympany, necrobacillosis of hoofs, leptospirosis, stomatitis, etc.

Whereas the treatment and healing of cattle is necessary to keep them in life, administering of antibiotics to these dairy animals is inevitable. Estimates of current and preliminary data from the veterinary structures indicate that the use of drugs is massive in dairy farms in our country. We can freely say that about 80% of the total amount of drugs that were given to dairy cattle is antibiotics; their types are presented in Fig5.

The aim of this study is to detect and to assess beta-lactam residues in Kosovo's milk, and to evaluate the antibiotics application on Kosovo's dairy cattle.

MATERIALS AND METHODS

To carry out this study over 80% of Kosovo's dairy farms were visited from January to August 2011. During this time a total of 127 raw milk samples were collected, of which 39 were collected during the winter season and the rest, 86, during the summer.

The samples were taken only from sick cows treated with Beta-lactam antibiotics at least two weeks prior to the start of collection.

The assesment has been carried out through a specific questionare which highlights and identifies problems with drug treatments of animals.

The samples have been analysed at laboratories of the Veterinerian Institue of Skopje using the Elisa method.

To examine the Beta-lactam residues in the raw milk samples the Elisa method made by Randox – England was used.

This method is one of the fastest and the most reliable of methods in antibiotic detection. The test is also easy to use, has a high sensitivity and a fast reaction.

To prepare the milk samples for the beta-lactam detection test, these reagents were applied:

a) a flushing puffery (concentrated) of 1 x 32 ml, by Randox-England

b) 6 types of standards,by Randox-England,

c) a conjugate (concentrated) of 3 bottles, by Randox-England,

d) one shot substrate of 1x15 ml, by Randox-England,

e) one bottle of preventive solvents, by Randox-England and f) redistilled water.

In this work all samples and standards applied on the microtite plate were duplicated. For the detection of beta-lactams the 6 applied standards follow these concentrations: a) 0 ng/ml; b) 0.46 ng/ml; c) 1.0 ng/ml; d) 5.6 ng/ml; e) 10.1 ng/ml dhe f) 58.9 ng/ml.

All the parameters are determined based on the criteria set out in conformity with the requirements and recommendations of Directive Regulation 96/23/EC [15].

For each batch of the analized samples, parametres that were set are a) selectivity, b) precision (accuracy), c) reproducibility, d) limit of detection and e) detection capability (CCβ) and their results were calculated using Excel program, which are presented in table no.1.

Table 1: The determined parameters of Elisa method for the analysis of beta-lactams residues in milk

Parameters	For beta-lactams
Range of concentrations	0.00-58.9 ng/ml
Detection capability (CCβ)	48.5 µg/kg
Reproducibility (precision)	6.28%
Calibration curve	Y = -0125 + 0.7744 lnX
Regression coefficient	0.879
IC_{50} (concentration at 50 % bounding)	0.65 ng/ml
Recovery (accuracy)	96.18 %
Limit of detection (LOD)	1.5 µg/ml
Repeatability	5.05 %

The read absorbance has the following formula: y = a + b*lnX; y – read signal expressed in% of optical density, X – concentration of the substance, and a and b – coefficients.

R2 value for beta-lactams must be at least 0.8278. Where as, Limit of Detection (LOD) as defined by the Elisa method for beta-lactam residues in the milk isdetermined by the formula: LOD = $X_{average}$ + 3SD, and it is 1.5 µg/ml.

In the Fig.3. shows the view of color changes from blue to yellow during the detection of antibiotic residues in milk samples. In wells without color the concentration level of antibiotic residues is higher.

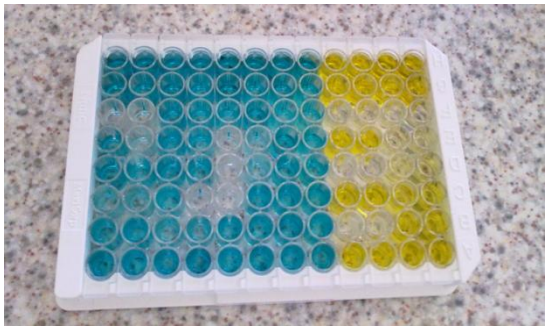

Fig. 3: It Shows the microtitre plate of Elisa screening method

RESULTS

Fig4. shows the percentage of the application of beta-lactams, sulfonamides, tetracyclines and other antibiotics. The study has proven that 64 sick cattle or 50.4% of the total were treated with beta-lactams, and this is shown with a highly significant statistical difference in relation to the use of other types of antibiotics ($\chi 2=134.0$; $p<0.01$). Sulfonamides rank the second most widely used antibiotics for the treatment of sick cattle in Kosovo.

Fig 5. Shows the percentage of cows treated with beta-lactams and other different types of antibiotics. The performed assessment shows that for the treatment of cattle with different diagnoses or with "health impaired conditions" 12 types of antibiotics are used, including betalactams.

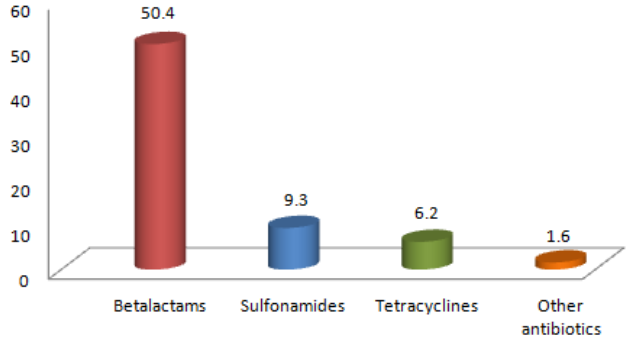

Fig. 4: It Shows the percentage of the application of beta-lactams, sulfonamides, tetracyclines and other antibiotics

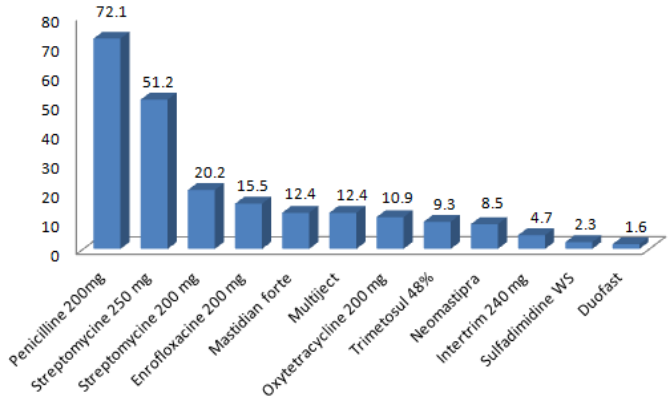

Fig. 5: It Shows the percentage of cows treated with beta-lactams and other different types of antibiotics

The most used drug is penicillin, 93 cows or 72.1% were treated with it, followed by streptomycin, 66 cows treated or 51.2%, and the rest were treated with other types of antibiotics.

Fig6. shows the percentage of beta-lactam levels in the milk of cattle. The assessment shows that from all analyzed milk samples, 11.6% of them contain beta-lactam residues in levels higher than the maximum limit allowed.

The maximum limit allowed is 1.5 µg/ml, whereas our study shows that in 11.6% from our total samples the residue level ranges from 2 µg/ml to 80 µg/ml.

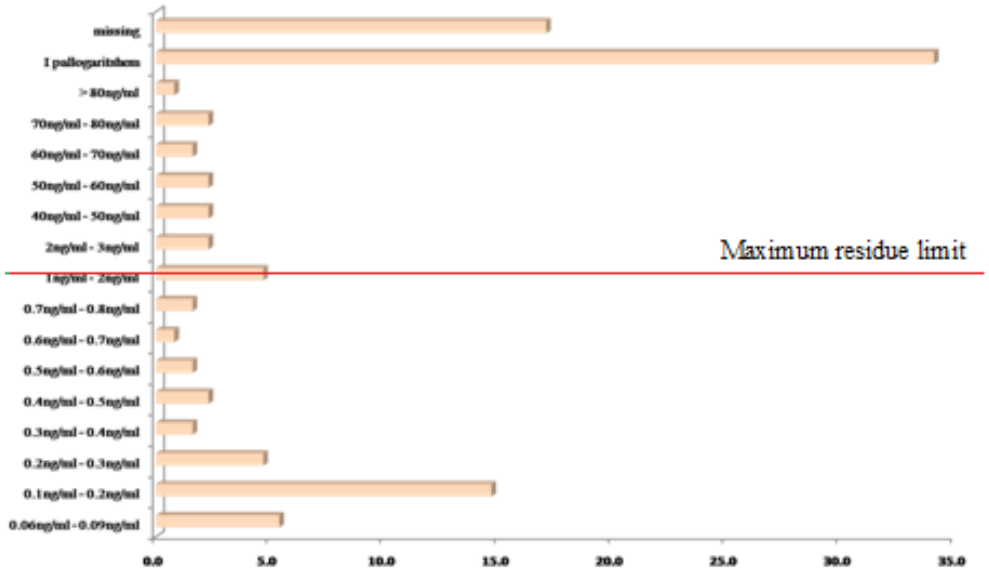

Fig. 6: It shows the percentage of beta-lactam levels in the cattle milk

However, in the majority of the samples the level of beta-lactam residues is quite low below the maximum limit, in some even as as low as it is impossible to measure.

Likewise, the Fig. 6. prove that beta-lactam level in the milk is lower when there is a gap in time between the antibiotics application and the milk sample analyses. The wider this gap the lower the level.

Fig7. shows the percentage of the ways of drug application. For the treatement of sick cattle the most prefered way of applying the drugs in the animal is the intarmuscular way. 73 cattle or 56% of the total were treated in such way. Then follow the ones treated intramamarly, being 40 cattle, or respectively 31% of the total.

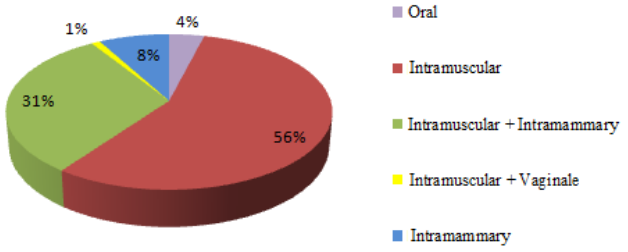

Fig. 7: It shows the percentage of the ways of drug application

DISCUSSION

During this study we have observed that, in Kosovo, cattle are primarily treated with beta-lactam antibiotics, where as secondarily with other types of antibiotics, such as: sulfonamides, tetracyclines, aminoglycosides and macrolides.

From the analysis of 127 milk samples the results show that 64 samples or 50.4% of the total are contaminated with beta-lactam residues.Whereas 15 samples or 11.6% of the total have surpassed the maximum limit allowed.

The study shows that 78 dairy cattle or 60% of the total were given the drug in an intramuscular way, whereas in 40 of them or 31% of the total the drug was inserted in an intrammamar way.

The rules and procedures of the treatment are not being followed, since antibiotics generally, should be given a minimum of 5 consecutive days, using the same dose every day and every time, in order for the medicine to have a curing effect as in humans so in cattle. It is clear that behind these negative occurrences in the treatment of cattle lies the ever growing motivation for profit-making and cost-reduction. In oder not to waste few days in milk production, not only farmers but also veterinarians themselves do not abide by protocols of treatment, applying instead the so-called 'short schemes of treatment' which not only helps not to impede their milk production but also saves them the incurring cost in medicines and general treatment of the cattle.

According to a study carried out in Turkey, by Kaja and Filazi (2010) from all analyzed samples only 1.25% were found to have been contaminated with beta-lactam residues.

Other study carried out in Italy, by Ghidini (2002), shows that out of 53 of the analysed raw milk samples 29 were found to be contaminated with beta-lactam residues, of which 27 have already surpassed the maximum limit allowed while the rest, 2, were on the brink of this limit.

Moats (1999) found 54 beta-lactam contaminated samples out of all his raw milk samples analyzed using his examination method.

Riediker et al.(2001) analyzed 549 raw milk samples through the examination method, resulting in 53 being contaminated with beta-lactam residues.

According to a report on milk purity and safety published by World Health Organization (WHO) and The Joint Expert Commitee on Food Additives (JECFA**)** the degree of antibiotic contaminated milk and dairy products in developed countries such as USA, Australia, UK and Scotland reached 7 -10 %, up to the year 1969. Whereas the following years it dropped to 0.5% in USA, 2.1% in Australia, 1.5% in UK and 3.4% in Scotland, the reason being the measures taken to combat the persistent contamination of milk. The same report suggests that the degree of contamination in undeveloped and developing countries could be higher than the permitted limit (Navratilova 2008).

Other study carried out by Demet et al.(1992) has resulted in 6 out of 50 analyzed milk samples being contaminated with beta-lactam residues.

Moreover, a study carried out in Turkey by Alkan (2007) analyzing 81 milk samples resulted in only two samples being contaminated with beta-lactam residues.

CONCLUSION

From the results of this study we can conclude that there are risks involved in the consumption of public milk. To reduce this risk it is of chief importance that a number of certain activities are implemented.

Above all the inspection controls on record keeping of the treated animals and proper medicine use during treatments must be strengthened.

Also, a reliable and rigorous system is needed to monitor the safe distribution and management of antibiotic and antimicrobial medicines.

Drugs for human use should vary from those for veterinary use, being distinguished through labels either for human or veterinary use only,because it can lead to the creation and development of antimicrobial resistance, which appears when a microorganism is capable of surviving the exposure to an antibiotic, and this comes from excessive use of antibiotics both of human and veterinary medicine.

Treatment of cattle does not follow an exact "protocol" for betalactam application; due to this, there is a need for development of these protocols and their implementation, and not leaving the treatment based on farmer "desire".

Moreover, the continuous development of food control laboratories is a must to enable milk examinations through sophisticated multi-residual methods.

Likewise, it is of great importance a launch of research initiatives on medicines' withdrawal period, especially on antibiotics, so that contamination of milk and dairy products with various residues, which pose a great risk to the public health, could be successfully combated.

Raising public awareness on food safety and healthy eating as well as promoting the marketing of healthy and organic products is another measure which could help in fighting food contamination.

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Author Contributions: Shehi was overseeing this research project, had full access of the data in this study, meanwhile, he takes responsibility for the integrity of the data.

Study Design: Ibraimi, Hajrulai, Murtezani.

Instruction on the use of instruments for the outcome measures used in this study: Ibraimi, Hajrulai.

Analysis and interpretation of data: Ibraimi, Mata.

Manuscript preparation: Ibraimi, Shehi, Murtezani.

Statistical Analysis: Mata, Ibraimi.

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