

**STUDY ON OXYTETRACYCLINE RESIDUES IN COW'S MILK SAMPLES
COLLECTED IN TETOVO, MACEDONIA FROM 2012 TO 2013****Mensur Kamberi, Kapllan Sulaj****Summary**

The objective of this study was to determine the oxytetracycline residues in cow's milk collected in farms of Tetovo in Macedonia. The cow's milk samples produced in this area are controlled applying qualitative analytical tests for oxytetracycline residues in 262 milk samples through specific ELISA test. After this control positive milk samples were kept in freezing conditions to be analyzed by high performance liquid chromatography (HPLC) method in order to perform qualitative evaluation of oxytetracycline. The quantitative control with ELISA confirmed that 5.3% (14/262) of milk samples were positive for oxytetracycline residues. From analytical check performed by HPLC was confirmed that 2.3% (6/262) of total milk samples had different values oxytetracycline residues. Referring to MRL for oxytetracycline in milk only 1.6% (4/262) of analyzed samples has been confirmed with values higher than this limit. The quantity values of oxytetracycline calculated after analytical check with HPLC in 1 liter milk ranged 65ug/l to 1300ug/l (ppb). These study results are showing cases of oxytetracycline residues with high doses of oxytetracycline in milk samples signaling for risk of public health in consumers in Tetovo and other regions of Macedonia.

Key words: residues, oxytetracycline, milk, cow, Tetovo.

Introduction

Milk is food exposing human with antibiotics residues causing many health problems. The group of oxytetracycline as antibiotic with broad spectrum is commonly used in treatment of animal infections. These antibiotics have ability to kill bacteria through affecting producing of proteins that are essential for their living. The oxytetracyclines are used in lactating and non-lactating dairy cows for many treatments such as enteritis, pneumonia, diphtheria, infections caused by chlamydia, genital infections, urethritis and other infections in animals. This antibiotic contributes to reduce and stop the spread of the infection (Bishop et al., 1994). In many cases, some strains of bacteria developed resistance to this antibiotic reducing its effectiveness for treatment of some types of infections in cows and other lactating animals (Grave et al., 1993). Even though this benefit for animal health oxytetracycline residues are having adverse effects on people causing allergy and building up of antibiotic-resistant organism in humans. Other negative effect of oxytetracycline residues are related to inhibition of starter cultures used to produce fermented milk products such as yogurt and cheeses (Alica et al., 2003).

The treatment of metritis and other genital infections are remaining main cause of having residues in cow's milk which had the incidence from 7 to 15 % (Lewis et al., 1997). Commonly observed disease conditions recorded in 2013 in the dairy farms mastitis in average incidence more than 48.2%. In 2012 authors are reported that 14 % of infections in farms have as cause by enteritis, even other types of diseases were recorded in 22% of farms (Alica et al., 2006). The other disease conditions such as dystocia, retained fetal membrane, metabolic problem and foot problem recorded in dairy farms in 18%.

More than 50% of the farmers interviewed utilized oxytetracycline, as injection and only small number of farmers were aware to control cow therapy for controlling mastitis (Forst et al., 1991).

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On more than 2/3 of the dairy herds surveyed, veterinarians, assistants and owners themselves were used to administer antibiotics for treatment of infection by routes of intramuscular, intramammary, intrauterine and per os. About of 10% of oxytetracycline in treatment of animal infection is used in intrauterine way (Grave et al., 1999; Navrátilová et al., 2009). For above reasons oxytetracycline in milking cows is commonly used leaving residues of this group in different concentration.

The milk with concentrations of antibiotic residues higher than the MRLs is not allowed to be used by consumers. Protection of consumers and risk reducing is achieved by effectively control of antibiotic residues in milk and therefore, regulatory authorities approved maximum residue limits (MRLs) for oxytetracycline in milk. These regulatory limits protect consumers from over-exposure to oxytetracycline above maximum residue limit (MRL) of 100ug/kg milk. EU countries have introduced in national residues monitoring programs the control for residues of oxytetracycline in cow s' milk used for human consumption. On this context Republic of Macedonia is realizing each year national residues monitoring plan according to EU specific legislation for residues control. Concerning to public health treatment with oxytetracycline residues are causing adverse effects on people exposed to this kind of antibiotics.

Materials and method

Study was carried out in 2013 in cattle farms located in Tetovo region in Macedonia. 262 raw milk samples were collected from milking cows in farms in Tetovo. All milk samples from different locations of cattle farms were kept in the refrigerator (4°C) and analytical check was performed in two days.

Use of ELISA for qualitative detection

For detection of oxytetracycline residues in raw milk samples collected from milking cows in Tetovo is used MaxSignal® Oxytetracycline ELISA Test Kit as competitive enzyme immunoassay for the qualitative and quantitative analysis of milk samples. The method is based on a competitive colorimetric ELISA assay. The volume of sample is added along with the primary antibody specific for the target drug as is prescribed in instruction for use. On this context, if the target is present in the sample, it will compete for the antibody; thereby it is ensured prevention of antibody from binding to the drug attached to the well. The secondary antibody, tagged with a peroxidase enzyme, targets the primary antibody that is complexed to the drug coated on the plate wells. The resulting color intensity, after addition of substrate, has an inverse relationship with the target concentration in the sample. The detailed use of MaxSignal® Oxytetracycline ELISA Test is performed according to instruction closed to kit box.

HPLC analysis for qualitative detection of positive samples

All positive samples confirmed by MaxSignal® Oxytetracycline ELISA Test are tested with HPLC to quantify the residue of oxytetracycline.

The HPLC procedure is completed using chemicals and material as: acetonitrile and methanol in HPLC grade; oxalic acid dihydrate Suprapur and Na₂HPO₄ heptahydrate; ethylene diamine tetraacetic acid (EDTA) disodium salt, citric acid monohydrate (Thermo Fischer Scientific) were of purity grade. Solid phase extraction (SPE) column Oasis HLB, 3 cc, 60 mg was purchased from Waters (Milford, USA). The vacuum unit for SPE was purchased from Supelco. The other hardware included an analytical balance (Kern, Balingen, Germany), a cooling centrifuge (Mechanika Precyzyjna, Poland), and a rotary vacuum evaporator (Büchi, Flawil, Switzerland), (Petkovska et al., 2006). For the qualitative and quantitative evaluation, the external standard method was

used. Each sample was analyzed in duplicates way at the least, every series containing a blank sample. Simultaneously, aliquots of the milk samples with the addition of standard solutions of known concentrations were measured. The detection and quantization limits were established based on the standard deviation of the blind test and the slopes of the calibration curves, repeatability was based on 20 parallel determinations and the recovery was based determinations of the milk sample with the addition of the solution of standards of known concentrations (50µg/l and 100µg/l). Basic statistical processing was done using the Unistat software, Version 5.1 (Unistat Ltd. 1998).

Results and discussion

Table 1. – MILK SAMPLES COLLECTED IN DAIRY FARMS AND POSITIVE CASE OF OXYTETRACYCLINE RESIDUES FROM 2012-2013 IN TETOVO, MACEDONIA.

No. farms	No. milk samples	Detection of oxytetracycline residues by ELISA (%)	Detection of oxytetracycline residues with HPLC (%)	Above MRL (100 µg/L) in %
43	262	5.3% (14/262)	2.3% (6/262)	1.6% (4/262)

Table 2. – QUALITATIVE EVALUATION OF OXYTETRACYCLINE IN COW S' MILK SAMPLES CONFIRMED BY HPLC

Milk samples	No. of milk samples confirmed positive (%)	No. of milk samples with values of oxytetracycline (40 µg/l -100 µg/l)	No. of milk samples values of oxytetracycline (100-1000µg/l)	No. of milk samples values of oxytetracycline (>1000µg/l)
1.West part of Tetovo	4 (1.5%)	2	1	1
2.Central part of Tetovo	5(1.9%)	4	-	1
3.South part of Tetovo	2 (0.7%)	1	1	-
4.East part of Tetovo	3 (1.1%)	3	-	-

The analytical control of raw cow s' milk samples performed with ELISA found oxytetracycline residues. The use of the MaxSignal® Oxytetracycline ELISA kit for quantitative evaluation of oxytetracycline residues is recommended for qualitative detection by many studies. All positive samples were checked with HPLC to make quantification of oxytetracycline residues. The levels oxytetracycline residues showed values above MRLs in 1.6% (4/262). Oxytetracycline was found in all positive samples in a concentration from 65-1300µg/l. Levels of oxytetracycline in 2 milk samples were above 1000 ug/l. The higher incidence of oxytetracycline residues is confirmed in central part of Tetovo with value 1.9%. A given sample was regarded as positive for oxytetracycline if its retention time and peak corresponded to that of the standard. Retention time was considered a reasonably unique identifying characteristic of a given samples (Ding and Mou; 2000; Cinquina et al., 2003; Petkovska et al., 2006). The area inscribed by the peak is proportional to the amount of substance separated in the chromatographic system. For calculation of the concentration of oxytetracycline, a reference standard of a known concentration had been injected in to the HPLC and concentration of the sample was extrapolated from the curve peak area. Several studies in Europe carried out for detection of oxytetracycline residues in raw milk reported values of incidence from 0, 1 % to 2, 9 % (Allara et al., 2001; Navrátilová et al., 2009). There are many studies confirming the low incidence from 0, 001%- 1.5%. In some cases in Germany, Netherlands and USA the incidence of level of oxytetracycline was higher than 5%. Some author are attributing it the genital infections in milking cows (Heeschen et al., 1996). Comparing of chromatograms of reference standards, oxytetracycline HCl and some samples those were positive for oxytetracycline from the dairy farms were performed in these study to detect level of this antibiotic in milk. The range for oxytetracycline residue level was 0µg/l to 2400µg/l (Grave et al., 1999).

Conclusions

- This study is confirming the risk of oxytetracycline residues in cow's milk produced in cattle farms in Tetovo. Even though the low incidence (1.6%) of samples with level of oxytetracycline residues above MRL, number of samples confirmed positive (5.3%) by screening test is significant factor of use of oxitetraxicline in cattle treatment.
- Some milk quantities produced by cattle farms of Tetovo are exposing the consumers of Tetovo with high level of oxytetracycline residues.
- For that reason, the Competent Authorities of Republic of Macedonia should take more care on antibiotic residues control on central and west part of Tetovo where confirmed higher values of oxytetracycline above the MRL.

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