


Levels of Oxytetracycline Residues in Cow's Milk Collected in Tetovo, Macedonia From 2013-2014			Veterinary Medicine
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Abstract			
<p>The evaluation of oxatetracycline residues in cow's milk in cattle farms in Tetovo, Macedonia was carried out from 2013 to 2014. The control covered 25 milking cows' farms analyzing cow's milk samples for level of oxytetracycline. This study was completed performing analytical control for detection of oxatetracycline residue in 135 fresh milk samples using qualitative ELISA test. The positive samples confirmed by ELISA were kept in refrigerated condition and later are analyzed by high performance liquid chromatography (HPLC). The laboratory detection by HPLC made quantitative evaluation of oxytetracycline residues in milk samples. Analytical control carried out by ELISA test confirmed presence of oxytetracycline residues in 4.4% (6/135) of raw milk samples. Further analytical check with HPLC evaluated all positive milk samples confirming different values of oxytetracycline per liter milk: 60ug/l, 90ug/l, 220ug/l, 260ug/l, 430ug/l, 1340ug/l (ppb). The found values of oxatetracycline residues in cow's milk samples are shown the possible risk for public health in Tetovo region.</p>			

Introduction

Oxytetraxieline is a broad spectrum antibiotic that is effective against a wide variety of bacteria. On this context, some strains of bacteria have developed resistance to this antibiotic, which has reduced its effectiveness for treating some types of infections (Heeschen et al., 1996; Lee, et al., 2000). Oxytetraxieline is widely used to treat infections caused Gram negative and Gram positive bacteria resistant organism in humans. This antibiotic can inhibit starter cultures used to produce cultured milk products such as yogurt and cheeses (Alica et al., 2003). For these reasons it is important effectively control of antibiotic residues in milk and therefore, regulatory authorities have enacted maximum residue limits (MRLs) for a number antibiotic agents in milk (EMEA; 1995). National control programs of veterinary drug residues in various animals and their products, including milk, are compulsory in all EC countries as well as in Macedonia (Council directive 96/23 EC). Oxytetracycline is used to treat many animal infections caused by pathogen bacteria manly in lactating and non-lactating dairy cattle. The effective treatment of bacterial infections such as enteritis, pneumonia, diphtheria, infections infection caused by chlamydia, genital infections, urethritis is achived by using oxytetraxieline.

Oxytetraxieline can also be used to treat other rare infections, such as those caused by a group of organisms called riketeie (eg Q fever). To ensure that the bacteria that cause infection are susceptible to oxytetracycline diagnostic test are carried out (Petkovska et al., 2006).

Oxytetracycline works by interfering with the ability of bacteria to produce proteins that are essential to them. This antibiotic stops the spread of the infection and the remaining bacteria are killed by the immune system or eventually die (Bishop et al., 1994). Some bacteria strains have developed resistance to this antibiotic, reducing its effectiveness for treating some kinds of infections (Grave et al., 1993). Regulatory limits protect consumers from over-exposure to oxytetracycline above maximum residue limit (MRL) of 100ug/l milk (Lewis, 1997; Alica et al., 2003). Concerning to public health treatment with oxytetracycline residues is causing adverse effects on people exposed to this kind of antibiotics. Detectable concentrations of antibiotic residues in milk supplies higher than the MRLs are not allowed to be used to by consumers.

One of main cause of having residues in cow s' milk is treatment of metritis and other genital infections with incidence ranged from 7% to 15 % (Lewis, 1997). The most commonly observed diseases recorded in the dairy farms are mastitis in average incidence until 30% (Alica et al., 2003). According to the results of other studies disease conditions such as dystocea, retained fetal membrane, metabolic problem and foot problem recorded in dairy farms are having the incidence above 15%. Other authors are reporting that more than 50% of the farmers interviewed utilized oxytetracycline, and only 5% of the farmers were aware of dry cow therapy for controlling mastitis (Forst et al., 1991). Administration of antibiotics using the routes of intramuscular, intramammary, intrauterine and peros in 52.9%, 30.9%, 8.8% and 14.7% of the farms respectively (Grave et al., 1999). For above reason oxitetraxycline in milking cows is commonly used and risk of residues of this group of antibiotics is higher.

Materials and method

Study was carried out in 25 cattle farms located in Tetovo district in Macedonia. 135 raw milk samples were collected in 1 year from different dairy farms in Tetovo. After collection of milk samples from different locations of cattle farms were kept in the refrigerator (4°C) and analytical procedure was performed within two days.

Use of ELISA for qualitative detection

The detection of oxytetracycline residues in raw milk samples collected from milking cows in Tetova is achieved by using 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. During the analysis, sample is added along with the primary antibody specific for the target drug. If the target is present in the sample, it will compete for the antibody, thereby preventing the 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. 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.

To perform HPLC procedure are used chemicals and material: Acetonitrile and methanol were of 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 2013 to 2014 in Tetovo, Macedonia.

No. farms	No. milk samples	Detection of oxytetracycline residues with HPLC (%)	Above MRL (100 µg/L) in %
25	135	4.4% (6/135)	2.9% (4/135)

Table 2. Levels of oxytetracycline in raw cow s' milk samples confirmed positive by HPLC

Milk samples	Qualitative evaluation by HPLC
1. West part of Tetovo	60 ug/l
2. Central part of Tetovo	90 ug/l
3. South part of Tetovo	220 ug/l
4. West part of Tetovo	260 ug/l
5. West part of Tetovo	430 ug/l
6. West part of Tetovo	1340 ug/l

Oxytetracycline residues in raw cow s' milk samples confirmed by ELISA test are tested to find out quantitative values for each positive samples as is showed in table 2. Many studies recommended the use of the MaxSignal® Oxytetracycline ELISA for quantitative evaluation of oxytetracycline residues. In our study we use this kit to detect the presence of oxytetracycline in milk. All positive cases were confirmed by HPLC quantifying levels of oxytetracycline residues. The levels of oxytetracycline in positive samples above MRLs are calculated to be 4/235 or 2.9%. Values of oxytetracycline found in all positive samples ranged from 60-1340µg/l (Table 2). Levels of oxytetracycline in 4 positive milk samples above MRL were respectively 220ug/l, 260ug/l, 430ug/l and 1340 ug/l. The positive milk samples were analyzed by HPLC for oxytetracycline quantification. 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). The area inscribed by the peak is proportional to the amount of substance separated in the chromatographic system. To get 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. Studies in Europe carried out for detection of oxytetracycline residues in raw milk produced by milking cows reported values of incidence from 0, 2 % to 3, 7 % (Grave et al., 1999; Allara et al., 2001). Some other studies are confirming the low incidence from 0, 01%- 1.5%. In some cases in Germany and USA the incidence of level of oxytetracycline was higher than 5% and the cause was attributed 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 1600µg/l (Grave et al., 1999).

Conclusions

The control for oxytetracycline residues in milk samples collected in dairy farms in Tetovo in Macedonia confirmed positive cases of oxytetracycline in 4.4 % of total samples confirmed by MaxSignal® Oxytetracycline ELISA Test. Performing analytical control with HPLC oxytetracycline residues above MRLs were evident in 4 samples or 2.9%. The most of milk samples originated from west part of Tetova confirming values of incidence of oxytetracycline above the MRL and the risk of oxytetracycline residues in cow's milk produced in this area.

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