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Monitoring Quinolones in Bovine Milk in Kosovo

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Running title: **Detecting the Presence of Antibiotics in Milk Using ELISA Method**

Abstract
The aim of this work is to monitor the presence of quinolones in bovine raw Milk in the Republic of Kosovo by analyzing samples during a one year period in 2015, in order to determine the possible misuse of quinolones by veterinary practices or farmers, for the protection of human health. The Food and Veterinary Agency as the Competent Authority is implementing the National Residue Monitoring Program including antibacterial substances. During the concerned period, samples were collected by veterinary inspectors in five Kosovo regions. Of the 181 samples collected, 127 (70.01%) showed the presence of veterinary drugs above the maximum residue level (MRLs) established by European Union and Kosovo legislation, and 54 samples or 29.83 % were in compliance. Based on these results, there is a need to significantly increase the number of official controls on farms and of private veterinary practitioners, as well as the number of samples tested. Non-compliant results need to be traced back, and be sent to an authorized laboratory for confirmation. The results also present a need for adoption and implementation of European Commission Decision 2002/657/EC.

Practical applications
Detecting and quantifying of antibiotics with ELISA is easy method to detect the presence of quinolones and other antibacterial substances in milk, which gives the orientation for further trace the possible misuse of quinolones.

Key words: ELISA, quinolones, milk, MRL

Introduction

Antimicrobials are used widely in the management of dairy cattle. Improper use for disease therapy and as growth promoting agents can result in residues in milk and dairy products and can contribute to the development of microbial drug resistance and the spread of resistant bacteria, including those with serious health consequences in animals (Stolker et al., 2007). In order to prevent the development of antimicrobial resistance the European and USA health authorities have established strict limits to the levels of these substances in raw materials and foodstuffs. The EU has established the safe maximum residue limits (MRLs) for residues of veterinary drugs in animal tissues entering the human food chain. The establishment of the MRLs in the EU is governed by Commission Regulation (EU) No. 37/2010. This regulation repealing the Council Regulation (EEC) 2377/90 and its amendments and the authorized drugs that can be used for therapeutic purposes in animals intended for food production. Therefore, accurate detection of low levels of antimicrobial drug residues in milk is of great importance for the dairy industry and also for farmers, to ensure that contaminated milk from individual cows is not consigned to the bulk tank (Mitchell et.al. 2003).

The residues of VMP present a potential risk to the consumer, particularly with the appearance of allergic reaction and interferences of intestinal micro-flora (Dewdney et al., 1991).

From a technological perspective, restudies of antimicrobial agents in milk can cause significant loss in fermented products, by inhibition of bacterial fermentation in the production processes of cheese and yoghurt (Molina et al., 2003).

ELISA is a common biochemical and analytical method available for the detection of veterinary medicine residues in food due to its high sensitivity, simplicity and ability to screen large numbers of small-volume samples (Watanabe et al., 2001, Zhang et al., 2007, Wang et al., 2009).

In order to monitor the possible misuse of veterinary drugs in Kosovo, the competent authority is implementing the National Residue Monitoring Program (NRMP); also Kosovo authorities transposed the EU Legislation into national legislation.

Materials and Methods

Sampling

A total of 181 raw milk samples were collected during 2015 at milk production farms, milk collection points and milk production establishments in five regions (Prishtina, Gjilan, Mitrovica, Peja and Prizren). Samples were stored at 4 – 8 °C, and each sample was divided into 5 sub-samples at the laboratory.

Sample preparation and Reagents

The sample is prepared and tested according the the manual and materials provided by the manufacturer of the kits made by BioScientific® (a PerkinElmer Company, Austin Texas, USA).

The reagents used for testing are also from kits provided by BioScientific® (a PerkinElmer Company, Austin Texas, USA), for Floroquinolones (MaxSignal® Floroquinolone Test Kit), Enrofloxacin (MaxSignal® Enrofloxacin Test Kit), Norfloxacin (MaxSignal® Norfloxacin Test Kit), Ciprofloxacin (MaxSignal® Ciprofloxacin Test Kit) and Danofloxacin (MaxSignal® Danofloxacin Test Kit).

The method is based on a competitive colorimetric ELISA immunoassay. The drug of interest has been coated in plate wells. During the analysis, sample is added along the primary antibody specific for the target drug. If the target is present in the sample it will compete for the antibody, preventing the antibody from binding to the drug attached to the well. Secondary antibody targeted with peroxidase enzyme, targets the primary antibody that is complexed to the drug coated on the plate wells. The resulting colour intensity, after addition of substrate, shows relationship with the target concentration on the sample. The wavelength at which the results were read is 450 nm.

Required materials: centrifuge, microtiter plate reader; incubator; vortex mixer; automatic and multichannel pipettes.

Necessary standards curve can be constructed by plotting the mean relative absorbance %, obtained from each reference standard (provided in the kit) against its concentration on logarithmic curve. A special program MaxSignal® ELISA Analysis program linked with Excel is used to read the results.

Results

The objective of this study was to monitor and analyze the residue levels of Quinolones in raw bovine milk. A total 181 milk samples were tested and 127 were positive in norfloxacin. Of these 40/47 (85.10%) from the Peja region were positive, 33/52 (63.46 %) from the Prishtina region, 22/29 (75.86 %) from the Gjilan region, 6/22 (27.27%) from the Mitrovica region and 26/31 (83.87 %)

from the Prizren region. The levels of norfloxacin measured ranged from 0.66 μg/kg to 18.33 μg/kg.

These results are shown in Figures 1 and 2 as numbers and percentages compliant and non-compliant as numbers and percentages.
The other quinolones tested for were below the MRLs according to EU regulations.

Discussion

As a MRL for Norfloxacin is not defined in Table 1 of Commission Regulation (EU) No. 37/2010, the presence of norfloxacin in milk is not permitted. This study showed the presence of this unexpected and non-permitted type of quinolone in raw milk in Kosovo.
In order to address this problem and to assure food safety and public health safety, the competent authority shall:
send all positive samples abroad to an authorized and accredited laboratory for confirmation purposes, increase the number of official controls, increase the number of samples tested, closely monitor and record the distribution of VMP'S among private veterinary practitioners, and adoption and implementation of European Commision Decision 2002/657/EC.

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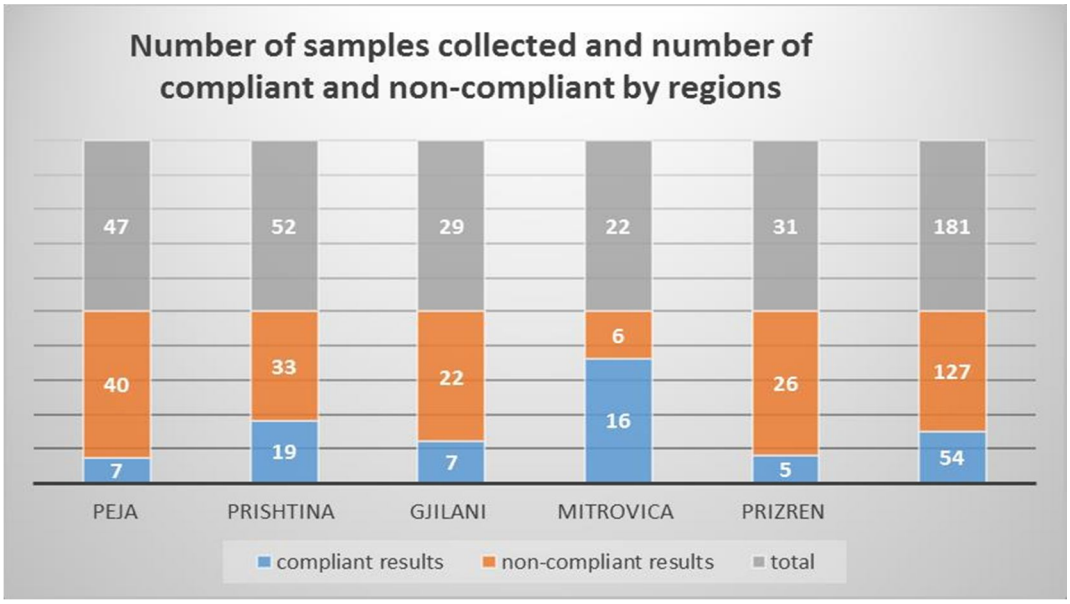

Figure 1. Number of collected and numbers of compliant and non-compliant by regions.

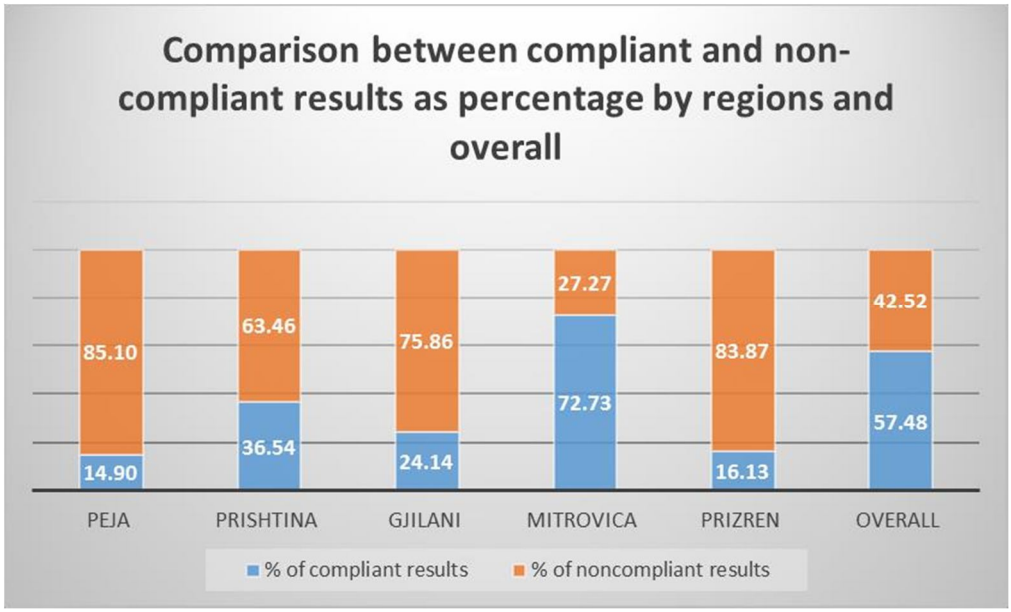

Figure 2. Comparison between compliant and non-compliant results as percentages by region and overall