Beta lactam antibiotics residues in cow's milk: comparison of efficacy of three screening tests used in Bosnia and Herzegovina

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Abstract

Beta lactam antibiotics are widely used in therapy of cattle, particularly for the treatment of mastitis. Over 95% of residue testing in dairies in Bosnia and Herzegovina is for Beta lactams. The aim of this paper is to compare the efficacy of three most common screening tests for Beta lactam residues in cow's milk in our country. The tests used in the study are SNAP β Lactam test (Idexx), Rosa Charm β Lactam test (Charm Sciences) and Inhibition MRL test (A&M). Study samples included: standardized concentrations of penicillin solution (0, 2, 3, 4, 5 and 6 ppb). In addition we tested milk samples from three equal size study groups (not receiving any antibiotic therapy, treated with Beta lactams for mastitis and treated with Beta lactams for diseases other than mastitis). Sensitivity and specificity were determined for each test, using standard penicillin concentrations with threshold value set at concentration of 4 ppb (Maximum residue level – MLR). Additionally we determined proportions of presumably false negative and false positive results for each test using results of filed samples testing. Agreement of test results for each test pair was assessed through Kappa coefficients interpreted by Landis-Koch scale. Detection level of all tests was shown to be well below MRL. This alongside with effects of natural inhibitors in milk contributed to finding of positive results in untreated and treated animals after the withholding period. Screening tests for beta lactam residues are important tools for ensuring that milk for human consumption is free from antibiotics residues.

KEY WORDS: β lactam residues; screening tests; cow's milk; Bosnia and Herzegovina

Bosn J Basic Med Sci. 2014;14(3):155-159.

INTRODUCTION

Residues are parent compounds and/or metabolites of biological and chemical substances routinely used in prescribed concentrations and in certain phases of animal production [1]. Antibiotics residues are the most common artificial inhibitory substances in cow's milk with negative impact on human health, processing and quality of the milk [2,3]. Besides infectious diseases agents common to animals and humans, contamination with environmental pathogens, antibiotics residues represent major problem in producing safe and good quality animal products. In many countries milk is continuously controlled for residues according to the legislative requirements. Numerous factors influence concentration of residues in milk, including individual characteristics, health of the animal, amount and type of applied antibiotics, quantity of milk production, method of antibiotics application etc. Parenteral applied antibiotics are excreted much faster through milk, while with intramamary application, residues are found for the longest period and in higher concentrations [2,3]. In our study we focused on Beta (β) lactam group of antibiotics, since they are widely used in therapy for bacterial infections in cattle, particularly for the treatment of mastitis. In addition as determined in this study all commercial dairies in Bosnia and Herzegovina (BiH) use antimicrobial residue tests, and 95% of testing is for β lactams. Aims of the study were: (1) asses sensitivity and specificity for the three most used ***screening*** tests for beta lactams residues in milk using standardized concentrations of penicillin, (2) evaluate efficacy of three most used screening test through proportions of presumably false positive and false negative results on samples from three animal study groups and (3) determine agreement of the results between all test pairs.

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Submitted: 12 May 2013 / Accepted: 16 April 2014

MATERIALS AND METHODS

Tests

We selected tests to be evaluated in this study according to the frequency of their use for screening for βlactam residues in cow's milk in B&H. Questionnaire was administrated to all laboratories, milk collectors and processing plants in the country regarding testing for residues in milk they applied during 2010. Results of the frequency of use of different tests for screening for β lactam antibiotics in milk were shown in Figure 1. Based on results shown in Figure 1, following tests were used in the study: SNAP β Lactam test (Idexx), Rosa Charm β Lactam test (Charm Sciences) and Inhibition MRL test (A&M). Even though all the tests used are intended for testing of bulk milk, they were applied in this study on individual animas samples, due to the fact that all test users in B&H apply them also on individual samples.

Samples

Samples of standardized concentrations of penicillin were prepared using standards produced by AiM, available as lyophilized milk containing penicillin G concentration of 0, 2, 3, 4, 5, and 6 ppb. Samples were liquefied using distilled and deionized water according to the manufacturer instructions. Concentrations of 2, 3, 5 and 6 ppb were represented with 10 samples each, five samples had concentration of 0 ppb and 20 samples contained 4 ppb (MLR for penicillin). Preparation and testing of the standardized concentrations of penicillin was done in Institute for food safety Zenica (regional authorized laboratory) and Food safety laboratory within the Veterinary faculty of the University of Sarajevo (national reference laboratory).

Milk samples were taken from three animal study groups, each containing 30 animals. The groups included:

- Group 1 - animals not receiving any antibiotic therapy during current and prior lactations,
- Group 2 - animals treated with β lactams for mastitis by intramamry application,
- Group 3 - animals treated with β lactams for diseases other than mastitis by intramuscular application.

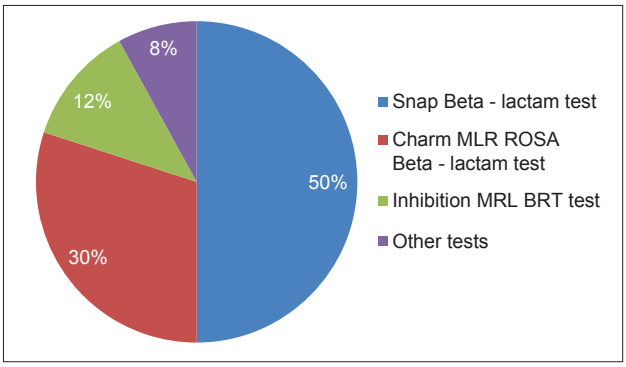

FIGURE 1. Use of different screening test for beta lactam residues by laboratories, milk collectors and processing plants in Bosnia and Herzegovina during 2010

Animals from the group 2 were tested within the withholding period, and day 1, 2 and 3 after the withholding period has passed. For treatment of cows with mastitis single application intramamary injector intended for use during lactation was used containing 100.000 i.u. procaine-benzyl penicillin. Withholding period for this product is 3 days. For treatment of animals for diseases other than mastitis intramuscular application of water suspension of penicillin with 150.000 i.u./ml was used. Withholding period for this product is days of therapy plus 5 more days. All sampled animals were Simmental breed, and according to the farm records in their second or third lactation. Appearance and nutrient content of all milk samples was approximately equal (data not shown).

After sampling milk was cooled at <5 °C and tested within 3 hours from sampling. Samples of milk were prepared for testing according to the instructions of test manufacturer. Preparation and testing of the field milk samples was done in the Institute for food safety Zenica and veterinary institute Teolab in Bijeljina (regional authorized laboratory).

For every test using results of testing of the standardized penicillin concentrations <4 ppb we determined specificity as proportion of negative results and overall samples with less than 4 ppb tested. Results of testing of the standardized penicillin concentrations equal or above 4 ppb were used for calculation of the sensitivity for every test as proportion of positive results and overall samples with equal or more 4 ppb tested.

Using results of the testing of standardized penicillin concentrations Kappa coefficient was calculated for every pair of tests used in order to evaluate agreement between tests [4]. Kappa coefficients were evaluated using Landis-Koch scale [5]:

- <0 – poor agreement
- 0.01-0.20 – slight agreement
- 0.21-0.40 – fair agreement
- 0.41-0.60 – moderate agreement
- 0.61-0.80 – substantial agreement
- 0.81-1.00 – almost perfect agreement

Test results from field milk samples originating from animals without antibiotics treatment, and animals sampled after withholding period were used to calculate proportions of presumably false positive results. Numerator of this proportion was number of positive results and denominator was number of tested animals in these two study groups. This proportion was referred as presumably false positive proportion since no confirmatory quantitative method was applied and therefore it was impossible to ascertain true if any concentration of β lactam residues in these samples.

Test results from field milk samples originating from animals treated with penicillin within withholding period were used to calculate proportions of presumably false negative results. Numerator of this proportion was number of negative results and denominator was number of tested animals in these two study groups. This proportion was referred as presumably false negative proportion since no confirmatory quantitative method was applied and therefore it was impossible to ascertain true if any concentration of beta lactam residues in these samples.

Since only Inhibition MRL test in addition to positive and negative results had option for suspect samples, all suspect results of this test were considered as positive for the purposes of data analysis.

RESULTS

Results of the testing of standardized penicillin concentration showed level of detection of 2 ppb for the Charm Rosaβ-lactam and Inhibition MRL test, and 3ppb for the Snap β-lactam test. Accordingly the Snap β-lactam test gave negative result for 52% of samples of standardized penicillin concentration bellow 4 ppb (specificity was 52%). Specificities of the Charm Rosa β-lactam test and the Inhibition MRL test were 24% and 20%, respectively (Figure 2).

None of the tests gave n egative results for standardized penicillin concentrations equal or above 4 ppb, resulting in 100% sensitivity for all three tests. Agreement between tests was assessed using Kappa coefficient for every test pair, interpreted according to the Landis-Koch scale (Table 1). Results of the testing of field milk samples from three study animal groups are shown in Table 2. Proportions of presumably false positive were given separately for group 1 (animals not treated with antibiotics) and group 2 (animals treated for mastitis) tested 1, 2 and 3 days after the withholding period. Overall proportion of presumably false positive results for each test for the above mentioned animals was given in Figure 3. All test results of the animas treated for mastitis within the withholding period were positive. Figure 4 shows proportion of presumably false negative results of animals treated with antibiotics for reasons other than mastitis within the withholding period.

TABLE 1. Kappa coefficient for every pair of the tests and its interpretation according to the Landis Koch scale

Test pair	Kappa	Interpretation of Kappa according to the Landis-Koch scale
Snap β-lactam and Charm Rosa β-lactam	0.512	Moderate agreement
Snap β-lactam and Inhibition MRL	0.508	Moderate agreement
Charm Rosa β-lactam and Inhibition MRL	0.901	Almost perfect agreement

DISCUSSION

Since β lactam antibiotics, dominantly penicillin, are most widely used in treatment of the bacterial diseases of cattle, therefore the tests for the detection of beta lactam residues are most widely used in control of milk for antibiotics residues. Even though they are useful tool for the prevention of use of residue contaminated milk, simultaneously they carry numerous disadvantages, firstly their ability to detect residues

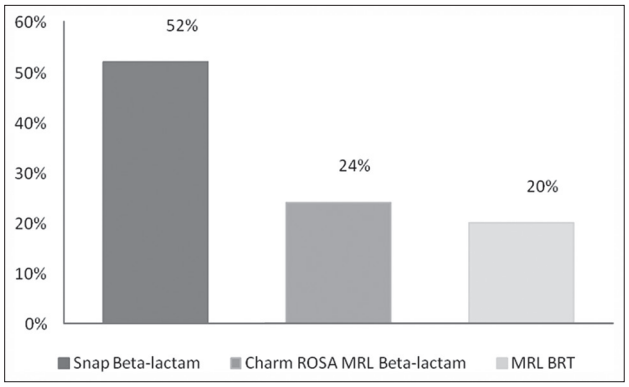

FIGURE 2. Specificity of the Snap β-lactam, Charm Rosa β-lactam and Inhibition MRL test assessed on samples of standardized penicillin concentration bellow 4 ppb

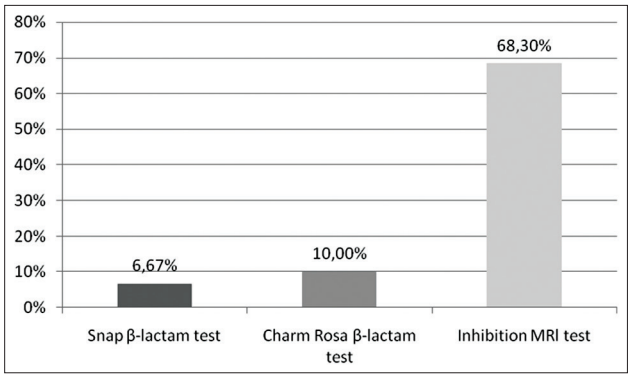

FIGURE 3. Overall proportion of presumablly false positive resuts (%) for each test for animals not treated with antibiotics (n=30), animals treated for masitis an samples 1, 2 and 3 day after the witholding period (n=90)

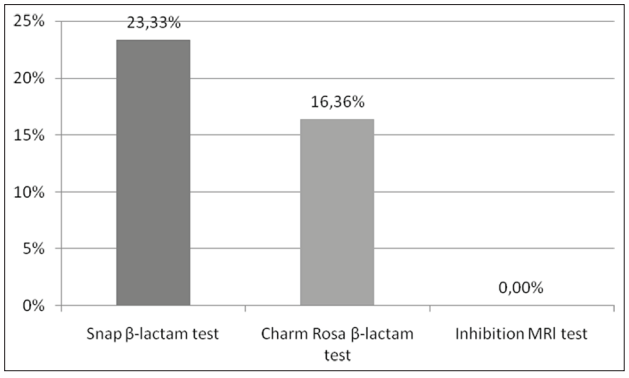

FIGURE 4. Proportion of presumablly false positive resuts (%) for each test for animals treated for reasons other than masititis durring the witholding period (n=30)

TABLE 2. Proportions of presumably false positive results (%) of the Snap β-lactam, Charm Rosa β-lactam and Inhibition MRL test for animals not treated with antibiotics and animals treated for mastitis day 1, 2 and 3 after the withholding period

Study group	Snap β-lactam test	Charm Rosa β-lactam test	Inhibition MRL test	n[1]
Group 1 – animals not treated with antibiotics	0.00%[2]	0.00%	63.33%	30
Group 2 – animals treated with antibiotics for mastitis day 1 after the withholding period	20.00%	40.00%	80.00%	30
Group 2 – animals treated with antibiotics for mastitis day 2 after the withholding period	20.00%	20.00%	80.00%	30
Group 2 – animals treated with antibiotics for mastitis day 3 after the withholding period	0.00%	0.00%	60.00%	30

[1] – Number of the animas in the group, [2] – Cells contain proportion of the presumably false positive results for each of the tests and for every animal group

bellow the maximum tolerated concentration. In our study we determined that residue screening tests for milk are applied in laboratories, milk collectors and processing plants, while milk producers themselves do not use them. However, legal requirements of the European Union, which B&H seeks to apply demand control of residues in milk on farm, through appropriate use of antibiotics, respecting the withholding period as well as testing of milk for the presence of antibiotics residues [6,7]. Most of the residue test users in B&H use test for β lactam residues while residue test that detect other antibiotics are neglected.

Lowest concentration of penicillin detected by the tests in our experiment was 2 ppb for the Charm Rosa β-lactam and Inhibition MRL tests and 3 ppb for the Snap β-lactam test, what is in accordance with the manufacturer specifications and other studies [8,9].

Perfect sensitivities and relatively low specificities of all three tests show determination of the test manufacturer towards protection of consumer's health rather than for the economic interests of the milk producers. Since according to the legal requirement producers are obliged to produce milk absolutely free from any residues, while MRL are set as the most tolerable concentrations, setting lowest concentration detectable by the test below required MLR seems justified and reasonable.

Differences in agreement between tests results were therefore influenced exclusively by the differences in tests specificities and chemical principle of residue detection, since all test had 100% sensitivity. Since Charm Rosa β-lactam and Inhibition MRL test had similar specificities (24% and 20%, respectively) agreement between these two tests was the highest. Higher specificity of the Snap β-lactam test influenced its agreement with both Charm Rosa β-lactam and Inhibition MRL test results. Based on these results it seems appropriate to combine test with lower agreement of results, in addition to no economic justification for the use of combination of tests that provide almost identical results.

Snap β-lactam and Charm Rosa β-lactam test gave negative results for all field samples of milk originating from animals in the Group 1 (no antibiotics treatment). In other study [8] samples of residue free milk were tested by the Snap β-lactam and Charm Rosa β-lactam test resulting in 2.73% and 7.27% of false positive results respectively. In our study, in contrast to these two enzyme based test, inhibition MRL test gave 11/30 suspect and 8/30 positive results in this group. High proportion of presumably false positive results for the Inhibition MRL test can be explained by natural inhibitors present in normal milk. Before testing we did not apply any procedure for the inactivation of theses inhibitors neither is this recommended by the test manufacturer or applied by test users in B&H. However in 1963 in study by Kosikowski and O'Leary [10] positive results of the inhibition tests were explained by natural inhibitors in the milk, since after heating milk at 82 °C for 5 minutes with aim to inactivate natural inhibitors, only 1 sample remained positive out of 11 samples without residues that were positive in initial testing by inhibition test. Other research on residue free milk samples with positive inhibition test results confirmed this finding [11-14]. Additionally, the Inhibition MRL test in testing standardized penicillin concentrations (without inhibitors) gave negative results for all samples with concentrations bellow level of detection.

In our study, presumably false positive results on all three tests, first and second day after the withholding period originated form the same animals. Therefore it is possible that these samples either contained residues above MRL or slightly below but reacted positive since level of detection of the tests is lower than MRL set at 4 ppb. Also these presumably false positive results particularly on the Inhibition MRL test may have risen due to natural inhibitors in the milk. In another research 87,5% of milk samples taken first day after the withholding period tested gave positive results on LacTek and Charm test due to the natural inhibitors and not due to the contained antibiotic residues [15]. Many studies show relatively high proportion of the positive results on the screening residue test after the withholding period [9-16]. Factors relevant to the individual animal and most commonly associated with residues in milk over the set MRL after the withholding period are: simultaneous treatment with different drugs and different application methods [15], constantly high somatic cells count, amount of milk produced, adjuvants of the applied drug [16], too long and excessive use of antibiotics [17]. Studies show higher rate of false positive results after the withholding period in animals with repeated mastitis [18]. Disease history for the animals in our study was not taken into the consideration.

Test results for the animals in group 3 (treated by antibiotics by intramuscular injection for reasons other than mastitis) gave surprisingly high proportion of presumably false negative results (testing done during the withholding period). These proportions were 23.33% and 16.66% for the Snap β-lactam and Charm Rosa β-lactam test respectively. We could not find any studies with similar results. Possible explanation of this finding may be application of lower than prescribed dose or error in farm records, so that the sampling was done either too soon (excretion of penicillin in milk not yet begun) or too late (withholding period was already over).

CONCLUSION

In summary, most of our results we were able to confirm with other relevant research. We prove significance of testing milk residue in cows but also different issues in interpretation of each of the test results. Our results would be more complete if we were able to investigate other parameters of the sampled milk such as somatic cell count, bacteria count, confirmation of the true residue concentration, particularly for samples with positive results after the withholding period. On the other hand all used test are intended for testing of the bulk milk, where milk from many animals is mixed. In case that milk from one or more animals contained antibiotics residues their concentration would be significantly reduced by the rest of the residue free milk. Applying and interpreting tests on the individual milk samples would therefore definitely influence proportions of false positive results.

DECLARATION OF INTEREST

The authors declare no conflict of interest.

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