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STUDY REGARDING THE IDENTIFICATION OF SOME ANTIBIOTIC WASTE IN TREATED COWS' MILK

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Abstract

The concept of prudent usage of the antibiotics supposes that their application should have the greatest effect on human and animal health and they should determine the weakest bacteria resistance to the antibiotic used. Among the 48 milk samples assessed, 4 samples (8,33%) were positive according to the test accomplished with the Ecotest device. After 10 minutes of incubation, 91,67% of the samples had enough lactic acid. The lactic acid determined acid pH and phenolphthalein in acid environment is colourless. The test tubes containing the milk from these samples stayed white (the colour of the milk). For the rest of the milk samples (8,33%), because of the presence of antibiotic waste, the active (microbiological) substance did not develop and, since there was no lactic acid, the pH in these test tubes is slightly alkaline or neutral and the phenolphthalein becomes pink. The device used is responsive enough to find the β-lactam antibiotics in milk and it may be used at the farm level. The antibiotic concentration according to the "screening" was under the maximum admitted limit (4 μg/l) and all of the 4 samples were "screen positive".

Key words*: antibiotic, contamination, treatment, waste.*

INTRODUCTION

Ideal tests offer positive results as close as possible to the "Maximum waste limit" (LMR), defined as interest levels. The tests offering positive results to values that are much higher than LMR are debatable. The tests offering positive results under LMR need an excessive number of samples necessary for confirmation.

There are several screening tests. These tests were assessed in diverse experimental conditions (Bishop et al., 1985; Macaulay and Packard, 1981; Seymour et al., 1988; Andrew et al., 2000).

Bishop et al. (1985) reported false positive results for some screening tests where milk from individual cows was used. The false positive results represent losses for the producers as the milk may be rejected for consumption (Cola & Cola, 2017). However, Macaulay & Packard (1981), reported a smaller incidence of the false positive results for three out of four screening tests assessed.

The screening tests were accepted because they reached the standards for low incidence of the false positive results but also of the false negative results (FDA, 1997). Even though the maximum limits for the waste in milk were established (MRL), some situations show that, in certain countries, the antibiotic waste contamination is still a problem (for example, Brazil: Martins-Junior et al., 2007; Bando et al., 2009; China: Bai et al., 2005; Bai & Huang, 2006; Kenya: Shitandi & Sternesjo, 2004).

Numerous studies regarding the antibiotic waste testing focus on liquid milk, so that little attention is paid to formula. The necessity to monitor the formula imports for a variety of potentially damaging substances becomes very important.

In this sens, Kneebone et al. (2010) tested the efficiency of IDEXX tests (IDEXX Laboratories Inc) for identifying antibiotic waste in 5 varieties of formula. The results suggest that the IDEXX tests (New Beta-Lactum and New Tetraidine IDEXX Snap test Kits) actually identify the waste in commercial formula samples (Nestle - 3 samples, Campina one sample and Regilait one sample) and they may be used for monitoring the antibiotic waste in formula reconstituted products.

Also, the rapid tests chosen to obtain results at the farm level proved to be very good for identifying the antibiotic waste in milk that was mixed from several animal species (Contreras et al., 1997; McEwe et al. 1996; Andrew, 2000).

The incidence of some false positive results in raw milk was correlated to several factors, including here the high levels of lactoferrin,

feeding, lysozyme, milk fat, milk protein and the number of somatic cells in milk (Carlsson et al., 1989; Van Eenennaan et al., 1993; Marin et al., 2020; Andrew, 2000; Bonea, 2013, 2020).

It is interesting that the performance of the testing devices of antibiotic waste different for the breeds of milk cows. Andrew (2000) finds a growth tendency for the false positive results for the tests used to assess milk from Jersey breed compared to the tests used to assess milk from Holstein breed.

The immunologic tests are methods that detect specific interactions between the antibody and the antigen. These tests are divided in two basic categories, either direct or indirect; measuring the primary reaction antibody-antigen or the secondary reaction antibody-antibody.

The application of these immunologic tests for the analysis of antibiotic waste is made on different devices: LFD (lateral leak device), flash drives, ELISA, RIA, SPR (O´Keeffe et al., 2003; Campbell et al., 2007; Haughey & Baxter, 2006).

The rapid tests monitoring the enzymatic activity for identifying the β-lactam antibiotic class are available and represent now a well-established technology.

The enzymatic tests are generally considered as qualitative techniques that detect the presence of specific chemical waste or that are based on changing the colour reaction by assessing the final point of the test (Wang et al., 2012).

MATERIALS AND METHODS

This study was accomplished at the Society for Milk Production S.C. Fenov Dolj, within the milk cow farm.

S.C. Fenov S.R.L. has got a genetic patrimony compose of 120 Holstein Friesian lactating cows. The Holstein Friesian breed is specialised for milk production, having an average to high body development and a spotted and black appearance. The potential for the milk production is 9510 litres per lactation. For milking the cows, the unit uses a Herringbone 6x5 room with 30 milking parlours.

During August-October 2021, 48 milk samples were taken from the quarters of 14 cows treated against mastitis with β-lactam antibiotics intramammary. The milk samples were taken after the waiting time expired and they were assessed for the presence of some antibiotic waste by means of a rapid test, using the Ekotest device and by means of a screening laboratory test where the preparation of standard milk solutions was made by diluting the solution of penicillin G stock in milk with no inhibitors up to 0.008 units/ml. concentration, and the Agar with the indicator cooled down at 60ºC and it was inoculated with a suspension of *Bacillus stearothermophilus*. 6 ml of this agar were dropped on Petri plates and they were left to solidify on a plane surface.

The antibiotic quantification was made by means of paper disks impregnated with milk with different antibiotic concentrations 0.25 x MRL; 0.50 x MRL; 1 x MRL; 1.5 x MRL; 2 x MRL on agar environment Müeller Hinton seeded with active substance (*Bacillus stearothermofilus* spores).

Each antibiotic concentration was 4 times replicated. All plates were incubated at 55ºC for 4 hours.

After incubation, the inhibition areas around the paper disks were measured by means of callipers (0.1 mm accuracy). The disk diameter is measured twice next to the inhibition and the average is calculated.

The areas having a diameter over 15 mm were considered as positive areas.

Separately, on 8 different Petri plates, paper disks with 13 mm diameters were placed, immersed in the positive milk samples identified by the EKOTEST device. The plates were incubated at 55ºC for 4 hours. After the incubation, the inhibition areas were measured.

The correlations between the antibiotic concentrations used and the diameter of the inhibition areas were analysed. The correlation coefficient was calculated by means of this calculation formula:

$$\partial = \frac{n \times \sum xy - \left(\sum x\right) \times \left(\sum y\right)}{\sqrt{n \times \sum x^2 - \left(\sum x\right)^2} \times \sqrt{n \times \sum y^2 - \left(\sum y\right)^2}},$$

where:

X = antibiotic concentration (changed into Log_{10})

Y = diameter of the inhibited area

The antibiotic concentration in milk was quantified by means of the following calculation formula:

$$\bar{y} = a + b\,\bar{x}$$

RESULTS AND DISCUSSIONS

Using the "screening tests" for identifying the antibiotic waste in every cow's milk is associated to reducing the incidence of this type of waste in raw material milk.

Identifying some antibiotic waste

A test for identifying the antibiotic waste should meet the following conditions: identifying all the components included in the definition of antibiotic waste; identifying the waste at concentrations under the maximum admitted limit (MRL).

Ekotest is a test used for determining the presence of antibiotics and inhibitors in cow milk. It is a rapid test of 10-12 minutes. Relatively cheap reagents are used and 6 samples may be simultaneously tested.

Out of the 48 assessed milk samples, 4 samples (8.33%) were positive according to the test achieved by means of the Ecotest device (Figure 1). After 10 minutes of incubation, 91.67% of the samples showed enough lactic acid.

The lactic acid determines acid pH and phenolphthalein in acid environment is colourless. The test tubes containing the milk of these samples stayed white (the colour of the milk).

For the rest of the milk samples (8.33%), because of the presence of antibiotic waste, the active (microbiological) substance did not develop and, since there was no lactic acid, the pH in these test tubes is slightly alkaline and the phenolphthalein became pink.

The device that was used is sensitive enough to find β-lactam antibiotics in milk and may be used at the farm level. The antibiotic waste amount was quantified in the laboratory.

The presence of antibiotic waste in cow milk after the waiting period expired indicates that some animals and some treatment factors may extend the antibiotic excretion into the milk.

Serious diseases influence the pharmacokinetics of the medicine and the waiting period should be adjusted.

The high-doses treatments that overcome the recommended doses extend the antibiotic excretion.

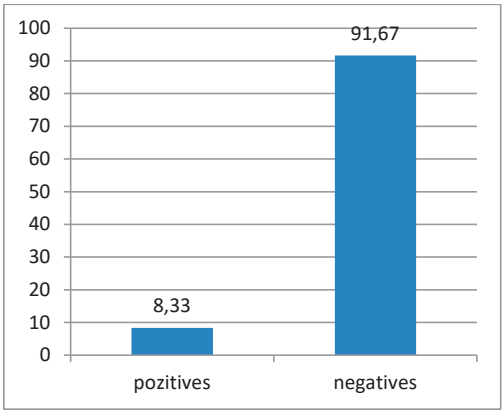

Figure 1 Assessing milk samples

Quantifying the antibiotic waste in the positive samples

Table 1. Determining the answer of the standard doses

Standard doses (UI/ml):	0.005	0.01	0.05	0.1	n = 4
Logarithm of doses (X lg_{10}):	-2.3	-2	-1.3	-1	$\sum(\bar{x}) =$ - 6.6
Square of the logarithm of doses X^2:	5.29	4.0	1.69	1.00	$\sum(\bar{x})^2 =$ 11.98 $(\sum \bar{x})^2 =$ 43.56
Average of the inhibition areas of the standard doses-mm $(\bar{y})$	15.4	-18.8	-23.7	-26.8	$\sum(\bar{y}) =$ 84.7
$X \times \bar{Y}$	-35.42	-37.60	-30.81	-26.80	$\sum \bar{x} \times \bar{y}$ = -130.63

$$b = \frac{n\Sigma\dot{x}\bar{y} - \Sigma\dot{x} * \Sigma\bar{y}}{n\Sigma\dot{x}^2 - (\Sigma\dot{x}^2)}$$

$$= 4\,\frac{[-130.63 - (-6.6 * 84.7)]}{4 * 11.98 - 43.56} = 8.37$$

$$a = \frac{\Sigma\bar{y} - b\Sigma\dot{x}}{n}$$

$$= \frac{[84.7 - (8.37 * -6.6)]}{4} = 34.98$$

$$\bar{Y} = a + bx$$

$$\bar{Y} = 34.98 + 8.37X$$

$$x = \text{antilog} * \frac{\bar{y} - a}{b}$$

The average diameter of the inhibition doses for the positive samples was the following:

Sample 1: $\overline{Y}$ = 15.6 mm

Sample 2: $\overline{Y}$ = 16.1 mm

Sample 3: $\overline{Y}$ = 15.8 mm

Sample 4: $\overline{Y}$ = 16.7 mm

The calculation of the antibiotic concentrations in the positive samples:

Sample 1:

$$x = \text{antilog}\frac{15.6 - 34.98}{8.37} = \text{antilog}_{10}\frac{-19.38}{8.37}$$
$$= \text{antilog} - 2.31$$
$$= 0.0049\ \text{UI/ml}$$

Sample 2:

$$x = \text{antilog}\frac{16.1 - 34.98}{8.37} = \text{antilog}\frac{-18.88}{8.37}$$
$$= \text{antilog} - 2.25$$
$$= 0.0056\ \text{UI/ml}$$

Sample 3:

$$x = \text{antilog}\frac{1.58 - 34.98}{8.37} = \text{antilog}\frac{-19.18}{-8.37}$$
$$= \text{antilog} - 2.29$$
$$= 0.051\ \text{UI/ml}$$

Sample 4:

$$x = \text{antilog}\frac{16.7 - 34.98}{8.37}$$
$$= \text{antilog}\frac{16.7 - 34.98}{8.37}$$
$$= \text{antilog} - 2.18$$
$$= 0.0066\ \text{UI/ml}$$

An international penicillin unit has 0.6 µg.

Sample 1= 0.0049 UI • 0,6 = 0.00294 µg • 100 ml = 2.94 µg/l

Sample 2 = 0.0056 UI • 0.6 = 0.00336 µg • 100 ml = 3.36 µg/l

Sample 3 = 0.0051 UI • 0.6 = 0.003.6 µg • 100 ml = 3.06 µg/l

Sample 4 = 0.0066 UI • 0.6 = 0.00396 µg •100 ml = 3.96 µg/l

Maximum admitted limit (MRL) = 4 µg/l

The antibiotic concentration after "screening" is under the maximum admitted limit and all of the 4 samples, even though the samples are "screen positive" (Figure 2).

The maximum limits of waste (MRL) are the waste levels accepted for food. The MRL levels show food safety and commercial standards. The medicine waste in animal products may be dangerous for the consumers' health. For every medicine, there is a waiting period for human protection.

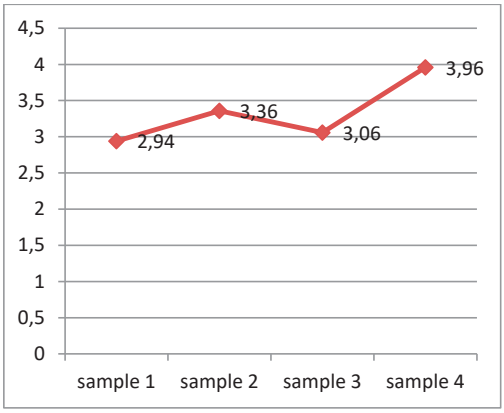

Figure 2. Antibiotic concentration (µg/l)

The waiting period is the time necessary for the antibiotic waste to reach concentrations under the tolerance levels. The maximum waste limits (MRL) stipulated by the European Union legislation guarantee the consumers' protection. Modern technologies may detect the antibiotic waste at level of part per billion (ppb).

This means that the milk dilution will never be enough to totally remove the antibiotic waste from milk. Using "screening" tests for identifying the antibiotic waste in raw material milk prevents that waste from entering the food chain. Each farm is responsible for preventing the milk from being contaminated. Using medication in a wrong way and abusing it for treating milk cows causes the contamination of the milk with waste above the established maximum limits (MRL).

Consequently, the milk becomes unusable for human consumption or for industrial processing. Moreover, the milk is also a component of other food products so that the antibiotic waste may contaminate those products.

The strategy of preventing the milk contamination with antibiotic waste should be based on correct procedures of using medication at the farm level. The presence of antibiotic waste in the cows' milk after the waiting period expired indicates the fact that some animals and some treatment factors may extend the antibiotic excretion into the milk. The test is quick (12 minutes), and the cost of a determination is small. The test identifies the inhibitory waste in milk under the "MRL" levels.

The sensitiveness of the ECOTEST method guarantees the fast identification of some

antibiotic waste in cow milk. The studied milk presents no risks for the consumers' health and some of its components, after the treatment of severe mastitis, influence the test for identifying the antibiotic waste.

CONCLUSIONS

Using the "screening" tests for identifying the antibiotic waste in raw material milk prevents it from entering the food chain. Preventing the milk contamination is the responsibility of every farm producing milk for human consumption.

The ECOTEST "screening" test that was used identified 8,33% positive samples out of the total samples. The test is rapid (12 minutes), and the cost of a determination is low. The test identifies the inhibitor waste in milk under the "MRL" levels. These levels were quantified in the laboratory.

The sensitiveness of the ECOTEST method guarantees the rapid identification of some antibiotic waste in cow milk.

The milk from S.C. Fenov S.R.L. presents no risks for the consumers' health.

After the treatment for serious mastitis, some milk components influence the test for identifying antibiotic waste. Among them, we may name the following: somatic cells, lactoferrin, lysozyme, free fat acids or sodium.

Using the antibiotic overdoses for treating sick animals should be associated to finding antibiotic waste after a waiting period.

We recommend testing milk from treated animals on the first day after the waiting period in order to identify the milk having antibiotic waste from animals with serious diseases or that had been overdosed. Research is necessary in order to find out the real prevalence of using overdoses on milk animals.

When we find the necessity to use overdoses of the same medicine, replacing it with a different antibiotic, used within the prescribed doses, may avoid the problem of antibiotic waste in milk.

Hygienically, the antibiotic waste and the contaminated substances should be as low as possible. The maximum limit of the waste in milk guarantees the consumers' protection, including for those at the end of the food chain (especially children) and offers protection from a link to the other against the accumulations that may appear in the human body.

Implementing activities of identifying and quantifying some antibiotic waste or some other harmful substance in milk within the HACCP program is feasible if the farm manager is trained and has enough information on this topic.

Controlling the risks during the primary processes of milk production determines the reduction of the contamination risk for raw material milk.

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