*Agronomy Research* **12**(3), 807–812, 2014

# Use of *Delvotest T* for Quantitative Estimation of β-lactam Antibiotic Residues in Waste Milk and for Evaluation of Thermal Treatment Efficiency – a Methodical Pilot Study

A. Sats*, H. Mootse, L. Lepasalu and V. Poikalainen

Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences, Department of Food Sciences, Kreutzwaldi 56/5, EE51014 Tartu, Estonia; *Correspondence: andres.sats@emu.ee

**Abstract.** The aim of this work was to study possibilities of using microbiological broad-spectrum inhibitor test (*Delvotest T*) for express estimation of antibiotic residues in bovine milk. For quantitative estimation the waste-milk samples were stepwise diluted (dilution array) by antibiotic-free milk until negative test result was achieved. Another objective was to evaluate heat treatment efficiency of waste-milk in order to degrade antibiotic residues in it. Heat treatment (in water bath) at 90°C was chosen according to references in literature. In order to study certain drug residue the waste-milk samples were collected at the next milking after medical treatment. Two different drugs were investigated: Carepen and Norocillin. Preliminary results indicated that the average concentration of antibiotic residue (active substance of drugs) exceeded European MRL (Maximum Residue Limit) 4,100 (± 2,408) and 13.0 (± 5.7) times respectively. The average heat treatment duration at 90°C (until negative test result of *Delvotest T*) was 15.0 (± 5.0) and 7.8 (± 1.5) hours respectively.

**Key words:** Waste-milk, Antibiotic residues, *Delvotest T*, Heat treatment.

## INTRODUCTION

It is widely known that improper use of antibiotics may lead to residues in milk, especially when withdrawal times are not respected. These residues can be dangerous for human health. They may cause allergic reactions, antibiotic resistance of pathogens etc. Antibiotic residues can also create a technological problem for industry production concerning bacterial fermentation processes in dairy products (Packham et al., 2001).

According to Statistics Estonia (www.stat.ee, 2014), the production of raw milk in 2012 was 721,200 tons. In addition to that, there is an estimated 13–19 thousand tons of raw milk containing antibiotic residue (AR). This milk is also known as waste-milk (WM), which is still an unused resource. For the use of that resource inactivation or degradation of AR is to be carried out. To control these processes estimation of antibiotic concentration in milk is of crucial importance.

Nowadays there are several receptor-based lateral flow assay tests employed routinely at the farm level and in the dairy industry because they are fast and simple to use. Microbiological test kits based on the inhibition of *Geobacillus stearothermophilus* are most frequently used for the screening analysis of milk in farms and dairy industries. According to Reybroeck and Ooghe (2012) the *Delvotest T* is a

new version of *Delvotest SP-NT* with an improved detection capability. It detects β-lactam (and many others) antibiotics efficiently in respect of actual Maximum Residue Limit (MRL) legislation (Commission Regulation (EU) No. 37/2010 and amendments). The important advantage of microbiological methods is that false negative samples can be almost excluded. Although it must be acknowledged that false positive results could occur if other than antibiotic inhibitors are present. Disadvantage of this method is that threshold level (MRL) of antibiotic in milk could be estimated only. For concentration studies expensive and sophisticated methods basing on chromatography should be used.

Thermal treatment is likely one possibility for antibiotics degradation in milk. There are several studies concerning thermal degradation of β-lactam antibiotics from a pharmaceutical aspect in aqueous solutions under different storage conditions, at different pH, etc. Also there are some studies about the effects of temperature on β-lactam antibiotics in foodstuffs (Moats, 1999; Hanway et al., 2005; Zorraquino et al., 2008; Roca et al., 2010, 2011, 2012).

Roca et al. (2011) investigated the effect of heating on the stability of β-lactams (penicillins and cephalosporins) in skim milk (skim milk powder was reconstituted to 10%). The results indicated that the conventional milk processing techniques causes minor losses in the concentration of these antibiotics and therefore do not prevent these antimicrobial substances from reaching consumers. Although it was clearly shown that milk sterilization at 120°C for 20 min had significant impact towards stability of β-lactams. Zorraquino et al. (2008) used a bioassay based method on the inhibition of *Geobacillus stearothermophilus* var. *calidolactis*. They studied three industrial heat treatments regimes. Of those classic sterilization (120°C for 20 min) showed convincingly highest level of heat inactivation of over 65% for penicillins although penicillin G concentrations in fortified milk samples were used only three times over MRL. However it can be concluded that duration of thermal treatment is a substantial factor concerning inactivation of β-lactam antibiotics.

Researches dealing with quantity of antibiotic residue in milk have been conducted mostly by spiking/fortifying milk (skim milk or commercial UHT milk) with antibiotic and the analysis of drug concentration performed by sophisticated laboratory equipment such as liquid chromatography etc. The aim of this work was to study actually treated cow's raw milk and to conduct a pilot experiment for investigation of a simple alternative for estimation of the quantity of (β-lactam) antibiotic residues in milk by *Delvotest T*. Additional goal was to scan thermal degradation of such milk.

## MATERIALS AND METHODS

The samples of raw milk for this study were collected from treated cow´s in Põlva and Hummuli farms and cooled down. Two veterinary drugs Carepen and Norocillin were under investigation in current study. Both drugs contained the same active substance (PBP – procaine benzylpenicillin) but concentrations and route of administration were different (Table 1). Carepen was used intramammary in case of mastitis and Norocillin intramusculary in case of leg disease. The samples (1.5 l per cow) were collected during next milking after the treatment (assuming that the milk

contains maximal possible concentration of antibiotic residue then). Experiments were conducted on the following day after collection of the samples.

**Table 1.** Description of drugs studied in current work, EU maximum residue limit (MRL) and detection limit (DL) of *Delvotest T*

| Drug | AS* | Route of administration | Drug admin. (ml) | AS in drug (mg $ml^{-1}$) | AS admin. (ml) | MRL (μg $kg^{-1}$) | DL of Delvotest T (μg $kg^{-1}$) |
|---|---|---|---|---|---|---|---|
| Carepen | PBP** | Intramammary | 10 | 60 | 600 | 4 | 4 |
| Norocillin | PBP** | Intramusculary | 40 | 300 | 1,200 | 4 | 4 |

*AS is active substance
**PBP is procaine benzylpenicillin

**Quantitative estimation of antibiotic by *Delvotest T***

The *Delvotest T* is a microbiological broadspectrum inhibitor test. To screen milk on the presence of antimicrobials it uses *Geobacillus stearothermophilus* var. *calidolactis* as a test organism. Microplates were incubated (floating on the water surface) in a waterbath at 64°C. After 3 h incubation, the color change of the pH indicator in the agar of the wells (yellow–negative, purple–positive) was recorded. For quantitative AR (antibiotic residue) estimation the waste-milk was diluted by antibiotic-free milk in order to achieve an array of different concentrations (dilution factors) of WM (Fig. 1 a). The 2.5% fat content commercial milk used as dilution environment was purchased from ordinary public store and tested by *Delvotest T* (no positive test results were found in current study).

**Table 2**. Example of dilution arrays used for quantitive estimation of antibiotic residue (AR). Bold represents AR below MRL (Maximum Residue Limit)

| Veterinary Drug | Carepen | | | | | |
|---|---|---|---|---|---|---|
| 1st dilution array | 500 | 1,000 | **3,000** | 5,000 | 7,000 | 9,000 |
| *Delvotest T* results | pos | pos | **neg** | neg | neg | neg |
| 2nd dilution array to refine results | 1,000* | **1,500** | 2,000 | 2,500 | 3,000* | |
| *Delvotest T* results | pos | **neg** | neg | neg | neg | |
| Veterinary Drug | Norocillin | | | | | |
| 1st dilution array | 10 | **30** | 50 | 100 | 150 | 200 |
| *Delvotest T* results | pos | **neg** | neg | neg | neg | neg |
| 2nd dilution array to refine results | 10* | **15** | 20 | 25 | 30* | |
| *Delvotest T* results | pos | **neg** | neg | neg | neg | |

* – dilution was made as control to ensure reliability of results

The dilution factor at which negative test result occurred in 1st dilution array was refined by 2nd dilution array and considered as detection limit (DL) of *Delvotest T* witch conveniently equals to MRL. Example of dilution arrays for quantitive estimation of antibiotic residue (AR) in case of Norocillin and Carepen are shown in Table 2. Negative results were taken into account only in case of clear colour change.

**Heat treatment**

For the heat treatment 50 ml sealed sample container was placed in thermostatic bath at 90°C. The 100 µl probes for detection of antibiotic residue (AR) were taken from the container hourly. Heat treatment was considered sufficient, and degradation of AR completed at a probe with negative test result (Fig. 1b). Objective was to establish the thermal degradation duration (TDD) at which negative test occurred (AR < MRL).

Roca et al (2011) found that the half-life of benzylpenicillin (PBP) at 90°C is 52 min. In current study this value was taken for bases and the predicted duration of thermal degradation (Table 3) was calculated by equations 1, 2, where PT is predicted time, $t^{1/2}$ is half-life of benzylpenicillin at 90°C (according to Roca et al (2011), 52 min), $n^{1/2}$ is count of $t^{1/2}$ (half-life of PBP), AR is estimated antibiotic residue and MRL is maximum residue limit of AR.

$$PT = n^{1/2} t^{1/2} \quad (1)$$

$$n^{1/2} = (\log AR - \log MRL)/\log 2 \quad (2)$$

**Figure 1.** *Delvotest T* results: a) estimation of concentration (dilution factors: 500–2,000) b) influence of thermal treatment duration (4–11 hours). Arrow points to the lowest dilution factor (1,500) and thermal treatment duration (10 hours), at which negative test occurred (AR < MRL). Originally colour photos are represented here in grayscale mode.

## RESULTS AND DISCUSSION

Table 3 presents estimated quantity of antibiotic residue (AR) in raw waste-milk and thermal degradation time of veterinary drugs (Carepen and Norocillin) AR. The difference between average AR concentrations of two drugs was remarkable (315 times). Significantly higher AR concentrations of Carepen can be explained by route of administration (intramammary or intramusculary). In addition to that the composition of drug (excipient) might have some impact to the results. Suggested 1st dilution arrays for quantity estimation of Norocillin and Carepen AR are presented in Table 2.

High standard deviation values concerning negative *Delvotest T* results (DF neg in Table 3), computational concentrations of AR (CC of AR) and thermal degradation duration (TDD) can be explained by differences in waste-milk quantity (WMQ) of particular milking. Negative correlation between AR concentration and WMQ also confirms that. High standard deviation value of TDD compared to PT of TDD can be

explained by cow´s individuality (milk production level, composition differences, etc.) which influences quantity of AR.

Initially just slight difference (in view of AR concentration) concerning the duration of thermal degradation of different WM samples as well as different drugs can be simply explained by exponential character of half-life degradation.

Remarkable correlation between computational concentration of AR and thermal degradation time supports the assumption that quantitative estimation of AR could be carried out by using *Delvotest T* on an array of diluted milk samples.

**Table 3.** Results of quantitative estimation and duration of thermal degradation of antibiotic residue in waste-milk

| Cow No | Drug | DF neg | CC of AR, µg kg$^{-1}$ | TDD till neg, h | PT of TDD*, h | WMQ, kg |
|---|---|---|---|---|---|---|
| 4,859 | Carepen | 1,500 | 6,000 | 10 | 9.2 | 16 |
| 5,364 | Carepen | 7,500 | 30,000 | 23 | 11.2 | 13 |
| 5,894 | Carepen | 3,500 | 14,000 | 19 | 10.8 | 12 |
| 6,805 | Carepen | 5,500 | 22,000 | 15 | 11.3 | 11 |
| 6,737 | Carepen | 2,500 | 10,000 | 14 | 9.8 | 12 |
| Average | | 4,100 | 16,400 | 15.0 | 10.24 | 12.8 |
| St dev | | 2,408 | 9,633 | 5.0 | 0.79 | 1.92 |
| Concentration correlation | | | | 0.82 | 0.84 | -0.45 |
| 439 | Norocillin | 5 | 20 | 6 | 2.0 | 13 |
| 123 | Norocillin | 15 | 60 | 7 | 3.4 | 6 |
| 6,790 | Norocillin | 20 | 80 | 10 | 3.8 | 9 |
| 5,979 | Norocillin | 15 | 60 | 8 | 3.4 | 10 |
| 5,286 | Norocillin | 10 | 40 | 8 | 2.9 | 16 |
| Average | | 13.0 | 52.0 | 7.8 | 3.1 | 10.8 |
| St dev | | 5.70 | 22.80 | 1.48 | 0.69 | 3.83 |
| Concentration correlation | | | | 0.83 | 0.80 | -0.65 |

* – Based on the half-life of benzylpenicillin at 90°C (Roca et al., 2011)
DF neg – Dilution factors of negative *Delvotest T* results
CC of AR – Computational concentration (CC) of AR based on DL=MRL (4 µg kg$^{-1}$)
TDD till neg – Thermal treatment (90°C) duration till neg. test result occurred
PT of TDD – Predicted time of thermal degradation duration (TDD)
WMQ – waste-milk quantity

Table 3 also presents predicted time (PT) of thermal degradation duration (TDD) based on the half-life of benzylpenicillin at 90°C described by Roca`s research group and estimated AR concentration results from our study. Compared to PT of TDD, the TDD of Carepen and Norocillin AR was significantly longer. This can be explained by the fact that the results were expressed only in case of clear colour change. So considering that, the actual concentration of AR might have been lower and/or the duration of AR degradation therefore shorter. It also might be caused by the composition of drug (excipient), route of administration, cow´s individuality, etc.

Further studies are needed to work out determined procedures for implementing proposed method in practice and for evaluation of it.

## CONCLUSIONS

For quantitative estimation of antibiotic residue in milk expensive and sophisticated laboratory equipment such as liquid chromatography etc. is normally used. The current pilot study demonstrates simple potential alternative for express quantitative estimation of antibiotic residues in bovine milk by applying *Delvotest T* to an array of diluted milk samples. This method is probably applicable in case of receptor-based lateral flow assay tests (testkits) also, which are employed routinely at the farm level and in the dairy industry because they are fast and simple to use. Preliminary conclusions of current study are: 1) proposed express method for quantitative estimation of AR in waste-milk and in heat treated waste-milk by *Delvotest T* is applicable; 2) the heat treatment study confirmed that procaine benzylpenicillin (PBP) in waste-milk can be degraded by long-term thermal treatment at 90°C.

ACKNOWLEDGEMENT. This work supports the investigation project 'Technological solutions for utilization of waste-milk and possibilities for reducing of its amount'. We are thankful to partners of the project and staffs of the Põlva and Hummuli dairy farms.

## REFERENCES

European Council. 2010. Council Regulation 37/2010 of the European Communities. Pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. *Off. J. Eur. Union.* L**15**, 1b–72.

Hanway, W.H., Hansen, A.P., Anderson, K.L., Lyman, R.L. & Rushing, J.E. 2005. Inactivation of Penicillin G in Milk Using Hydrogen Peroxide, *J. Dairy Sci.* **88**, 466–469.

Moats, W.A. (1999). The effect of processing on veterinary residues in foods. Advances in experimental medicine and biology Volume: **459**, 233–241.

Packham, W., Broome, M.C., Limsowtin, G.K.Y. & Roginski. H. 2001. Limitations of standard antibiotic screening assays when applied to milk for cheesemaking. *Aust. J. of Dairy Technol.* **56**(1), 15–18.

Reybroeck, W. & Ooghe, S. 2012. Validation report of the Delvotest T. January 6th 2012, 1–13.

Roca, M., Castillo, M., Marti, P., Althaus, R.L. & Molina, M.P. 2010, Effect of Heating on the Stability of Quinolones in Milk *J. Agric. Food Chem.* **58**, 5427–5431.

Roca, M., Villegas, L., Kortabitarte, M.L., Althaus, R.L. & Molina, M.P. 2011. Effect of heat treatments on stability of β-lactams in milk, *J. Dairy Sci.* **94**, 1155–1164.

Roca, M., Althaus, R.L. & Molina, M.P. 2012. Thermodynamic analysis of the thermal stability of sulphonamides in milk using liquid chromatography tandem mass spectrometry detection. *Food Chem.* **136**(2), 376–383.

Statistics Estonia. *http://pub.stat.ee/px-web.2001/Database/Majandus/13Pellumajandus/06Pellumajandussaaduste_tootmine/02Loomakasvatussaaduste_tootmine/02Loomakasvatussaaduste_tootmine.asp.* Accessed 10.1.2014.

Zorraquino, M.A., Roca, M., Fernández, N., Molina, M.P. & Althaus, R.L. 2008. Heat inactivation of beta-lactam antibiotics in milk. *J. Food Prot.* **71**, 1193–1198.