



# **Prevention of antibiotic residues**

*Appropriate management of antibiotic treatment of  
cows in automatic milking systems*

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# Prevention of antibiotic residues

## *Appropriate management of antibiotic treatment of cows in automatic milking systems*

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## Abstract

In conventional milking extended excretion periods of residues in milk exceeding the indicated withholding period are seldomly observed after antibiotic treatment of cows. Limited information is available if milking frequencies in automatic milking (AM) systems deviating from the regular milking times twice per day influence the excretion time of residues in milk. Therefore the present study was performed as part of the workpackage 5 "Prevention of antibiotic residues" within the European research project "Implications of the Introduction of Automatic Milking on Dairy Farms". In a previous study the excretion of antibiotic residues in milk of healthy cows in dependence on milking frequency was reported (D11).

In this report results of the second part of the investigation performed on cows with clinical mastitis are presented. For the treatment trials an udder injector containing cefquinome was selected, because relatively long excretion periods and large variation of excretion time were observed after application of this drug to cows without clinical symptoms of mastitis.

16 cows with 22 cases of acute clinical mastitis were included. In 17 cases cows were milked two times per day with intervals of 10 and 14 hours. In 5 cases cows were milked less frequently with intervals of 16 hours. One udder quarter per cow was treated at three successive milking times except for 2 cows in which 2 udder quarters were treated at the same time, a third cow received additional intramuscular treatment with the same antibiotic. In none of the cows the withholding period for milk of 120 hours was exceeded. In general, the excretion times were shorter than in a worst case experiment with repeated treatment of 4 udder quarters in healthy cows.

Different approaches for determination of withholding periods were applied. In contrast to the findings in healthy cows the milking frequency was of no significant influence on excretion time of cefquinome in milk. Only milk yield had a significant influence on excretion of residues in milk leading to shorter excretion times in cows with higher average daily milk yield during the experimental period.

From calculations based on results from excretion studies in healthy cows it can be concluded that the highest risk for bulk milk contamination due to carry over of milk from treated cows is for penicillin. The demands on automatic cleaning systems after milking of treated cows are rather high. In an experiment with 3 cefquinome treated cows no carry over of this compound was found into the milk of the next cow milked at the same place after performing a short cleaning cycle.

From the results of these studies and from the available literature recommendations are given for management of antibiotic treatment of cows in AM systems regarding farmer, veterinarian and AM manufacturer.

## Table of contents

<b>1</b>	<b>Introduction</b> .....	<b>1</b>
<b>2</b>	<b>Literature review</b> .....	<b>2</b>
2.1	Reasons for residues of antibiotics in bulk tank milk .....	2
2.1.1	Conventional milking.....	2
2.1.2	Automatic milking.....	2
2.2	Influences on excretion of antibiotic residues in milk .....	3
2.2.1	Milking frequency.....	3
2.2.2	Udder health status .....	3
2.2.3	Excretion from untreated quarters .....	5
2.2.4	Extra label use of antibiotics .....	5
2.2.5	Other factors influencing the excretion of residues in milk.....	6
2.3	Risk of bulk tank contamination .....	6
<b>3</b>	<b>Methodology and study materials</b> .....	<b>8</b>
3.1	Set up of treatment experiments .....	8
3.1.1	Parameters for characterization of cows .....	8
3.1.2	Milking frequencies.....	9
3.1.3	Sampling.....	9
3.1.4	Drug.....	9
3.1.5	Treatment intervals.....	9
3.2	Analytical methods.....	10
3.2.1	Udder health.....	10
3.2.2	Quantitative detection of antibiotic residues - HPLC methods.....	10
3.3	Determination of withdrawal time .....	10
3.3.1	Pragmatic approach.....	10
3.3.2	Time-to-Safe-Concentration (TTSC) method .....	11
3.3.3	Regression model.....	11
3.4	Analysis of variance.....	11
3.5	Efficiency of short cleaning cycle to prevent carry over of antibiotic residues .....	12
<b>4</b>	<b>Results</b> .....	<b>13</b>
4.1	Status of animals .....	13
4.2	Udder health.....	14
4.3	Excretion of residues in milk .....	15
4.4	Withdrawal time.....	17
4.5	Analysis of variance.....	23
4.6	Efficiency of short cleaning cycle to prevent carry over of antibiotic residues .....	24
<b>5</b>	<b>Discussion</b> .....	<b>25</b>
5.1	Udder health status .....	25
5.2	Influence of milking frequency.....	25
5.3	Total amount of drug excreted via milk .....	27
5.4	Dosage of applied drug .....	27
5.5	Efficiency of short cleaning cycle to prevent carry over of antibiotic residues .....	28
<b>5</b>	<b>Conclusions</b> .....	<b>29</b>
<b>6</b>	<b>References</b> .....	<b>31</b>
	<b>Acknowledgements</b> .....	<b>34</b>
	<b>Abbreviations</b> .....	<b>35</b>

## 1 Introduction

The main causes for inhibitors in bulk tank milk are associated with intramammary treatment of dairy cows due to clinical mastitis. In a French investigation they accounted for 64 % of positive results in microbial inhibitor tests (Fabre et al., 1995). Reasons for contamination of bulk milk were management failures like accidental milking of treated cows, not regarding prescriptions for dosage, route of administration or duration of the withholding period. A smaller number of positive findings was associated with dry cow therapy (24 %) or treatment of other diseases (11%). Similar problems are to be expected with automatic milking (AM) although human errors that are in a large percentage attributed to lacking information of temporarily employed milking personnel may be limited with AM.

One aspect that is especially linked to AM is the influence of different milking frequencies on the excretion of antibiotics in milk of dairy cows. In AM shorter milking intervals may occur due to more frequent milking as well as prolonged intervals when cows do not visit the AM system voluntarily. In the first part of this study (D11) experiments were performed on excretion of antibiotic residues in milk under conditions of simulated AM in healthy cows. Results from experiments in healthy cows are more easily to compare because influences like severity of disease or type of pathogens are excluded.

In our study 4 udder injectors containing 6 different antibiotics were tested in healthy cows and a significant influence of milking frequency on the excretion time was found in 3 drugs containing 4 different antibiotics. The excretion of residues in milk was prolonged with decreasing milking frequency.

In order to determine if results from healthy cows can be transferred to cows with clinical mastitis further studies were carried out. This was originally planned with an AM system, but carry over of antibiotics occurred in the automatic sampling device, because the tube for filling samples was not integrated into the cleaning process that is performed after automatic milking of cows. Therefore studies were performed under experimental conditions with milking frequencies simulated like in AM.

In this second part of the study one intramammary drug was tested in cows with naturally occurring clinical mastitis. The results are presented in this report. Cobactan<sup>®</sup> LC was selected for these experiments because long excretion times and large variability between individual cows were observed for this drug in healthy cows. In total 16 cows with 22 cases of naturally occurring clinical mastitis were treated. In 17 cases cows were milked at regular milking times with milking intervals of 10 and 14 hours. In 5 cases cows were milked every 16 hours. The time necessary for antibiotic residues in milk to fall short of maximum residue limits (MRL) fixed according to EU Regulation 2377/90/EEC was determined for the two groups.

In addition, one experiment was performed to evaluate the efficiency of the cleaning procedure used in AM systems in order to prevent carry over of antibiotic residues.

Based on the results from our studies and on information from the literature recommendations will be made for management of cows in AM systems after treatment with antibiotics.

## 2 Literature review

### 2.1 Reasons for residues of antibiotics in bulk tank milk

#### 2.1.1 Conventional milking

The frequency of positive inhibitor tests on bulk tank milk samples is rather low, ranging between 0.03 and 0.14 % of positive results in microbial inhibitor tests in European countries in 2000 (Suhren, 2002).

In most cases of inhibitor positive bulk tank milk samples the causes are related to management failures. According to an investigation by Schällibaum (1990) in Switzerland in the years 1986-1988 the main reasons for contamination of bulk tank milk were delivery of milk from treated cows during the withholding period, insufficient information flow between milking personnel, failures during milking, treatment failures or milking of dry cows after antibiotic treatment at drying off. These reasons accounted for 36.5 % of positive samples. Other major reasons were wrong milking order of cows (29.7 %) and insufficient cleaning of milking cluster or milking installation (29.3 %). Only in 3.1 % prolonged excretion of residues in milk was assumed leading to concentrations above the MRL after the end of the withholding period. 1.4 % were due to veterinary error.

In an investigation in France most of these causes were associated with intramammary treatment of dairy cows due to clinical mastitis (Fabre et al., 1995), accounting for 64 % of positive inhibitor tests. As the main reasons for contamination of bulk milk management failures like accidental milking of a treated cow, not regarding prescriptions for dosage, route of administration or length of the withholding period were observed. A smaller number of positive findings was associated with dry cow therapy (24 %) or treatment of other diseases than mastitis (11%). Similar findings have been reported by Hogeveen et al. (2000).

With automatic milking human errors like accidental milking of treated cows are expected to be limited, but other failures associated with treatment or not regarding withholding periods are not influenced by milking technology.

#### 2.1.2 Automatic milking

Rasmussen and Justesen (2003) reported a significantly higher frequency of bulk tank samples tested positive for inhibitors in herds with AM systems compared to conventional milking between 1998 and 2003. Although the percentage of positive samples decreased over time the number in 2002 was still 5 times higher (0.48 %) than in herds working with conventional systems (0.09 %). The highest percentage of positive samples was on farms working 90-180 days with AM systems.

The main reason for positive samples on AM farms was late or no reporting of treated cows to the AM system which corresponds to management failures in conventional milking. Technical reasons were only found in 4 out of 25 cases. Only in 3 cases no reason could be found which also corresponds to the percentage of positive inhibitor tests for which no reason could be found in conventional milking (Fabre et al., 1995).

One other reason for residues of antibiotics in milk might be the insufficient cleaning of milk contact surfaces after milking of treated cows. Pallas (2002) observed positive results of inhibitor tests in 6 (27 %) of 22 milk samples when milk of cows milked directly after cows treated with antibiotics and the following automated cleaning cycle was sampled. Positive samples were mainly detected when the treated cow was milked within 12 hours after treatment with antibiotics or when more than one udder quarter was treated. High concentrations of antibiotic residues in milk could be expected in these cases although the antibiotics were not quantified. Antibiotics used for treatment were cloxacillin, neomycin/penicillin (both intramammary treatment) and tylosin (intramuscular application). No positive bulk tank samples were observed during this investigation which is an indication that carry over of residues occurred only at a low level.

## **2.2 Influences on excretion of antibiotic residues in milk**

### *2.2.1 Milking frequency*

Limited and contradicting information is available on the influence of milking frequency on excretion of antibiotic residues in milk. Investigations were mainly focused on more frequent milking after treatment of clinical mastitis in order to determine the effect on therapeutic concentrations of antibiotics.

Schluep and Heim (1980) found slightly different concentrations of cefacetrile or penicillin resp. in milk of healthy cows in dependence of interval between treatment and milking, but this did not influence the duration of excretion in milk. When quarters were milked out at 4 additional times within the first 10 hours after treatment no difference in excretion of cefacetrile in milk was found compared to quarters of the same cow milked only at regular milking times twice per day. In contrast, Hamann and Heeschen (1995) reported lower concentrations of cefoperazone in milk of cows milked in intervals of 4 hours after intramammary treatment compared to cows milked after 12 hours.

According to Henschelchen and Walser (1983) the excretion time of procain-penicillin G or oxytetracycline resp. in milk was significantly shorter when cows were stripped 5 times at 2 hour intervals after treatment compared to cows milked at regular milking times. From the concentrations in milk it was concluded that already 2 hours after intramammary application the major part of the antibiotic is distributed within the udder and is not milked out with the first stripping.

### *2.2.2 Udder health status*

It has to be regarded that in studies on excretion of residues the udder health status is not always well defined. If no further explanation is given cows usually are referred to as healthy when no clinical symptoms of mastitis were detected.

After intramammary treatment Edwards (1964) found irregular distribution of penicillin in udder quarters during later stages of lactation. This was explained by an increase in connec-

tive tissue and shrinkage of ducts and alveoli. Similar findings occurred in cows with mastitis due to inflammatory changes going along with leukocytic invasion and partial occlusion of ducts. Higher concentrations and longer retention of penicillin were found in milk of quarters with chronic mastitis compared to healthy quarters. This was caused by increased fibrous tissue leading to poor distribution and absorption of penicillin.

Anderson et al. (1998) investigated the excretion of amoxicillin and penicillin G resp. in cows with clinical mastitis. Antibiotics were not detected in milk after the end of the withholding period, but one sample with SCC exceeding  $22\,000 \times 10^3/\text{ml}$  reacted positive in a microbial inhibitor test. No antibiotics were detected in this sample by liquid chromatography although the detection limit was lower than that of the screening test.

Schluep et al. (1979) observed that the elimination of cefacetrile was slightly retarded in experimentally infected quarters when a slow release formulation was applied for intramammary treatment, but no differences were detected between infected and healthy quarters when a quick release formulation was used.

After intravenous application of high dosages of ceftiofur in cows with induced *Escherichia (E.) coli* mastitis significant longer excretion of ceftiofur in milk was observed compared to healthy cows (147 hours versus 1.3 h) (Erskine et al., 1995). In milk samples of all 4 cows with mastitis ceftiofur was detected whereas this compound was only found in milk of one of 4 healthy cows. For ceftazidime similar concentrations in milk from healthy cows and cows with clinical mastitis were determined (Rule et al., 1998). For cefotaxime concentrations in milk of healthy quarters was only higher at milking one hour after treatment, but no differences between healthy and diseased cows were found 12 hours after intramammary application. For cefoperazone significantly higher concentrations were found in milk from quarters with subclinical mastitis than from quarters with clinical mastitis at 16 to 24 hours after intramammary administration (Zurich et al., 1993). No significant differences were found between concentrations in milk from healthy quarters and quarters with subclinical mastitis after udder treatment with cefoperazone or cephradine. Concentrations of cefoperazone above  $0.5 \mu\text{g}/\text{ml}$  were only found at 8 hours after application and no significant differences were observed between quarters with subclinical and clinical mastitis and healthy quarters.

For the macrolide antibiotics erythromycin and spiramycin lower concentrations were found in mastitic milk with  $\text{pH} > 7.2$  than in milk with  $\text{pH} < 7.2$  after intramammary or intravenous application (Nouws and Ziv, 1979). The excretion period in milk was shorter with more severe abnormalities of milk. These findings were consistent with those from Schällibaum et al. (1981) who reported lower concentrations in mastitic milk than in milk of healthy cows after intramammary application of spiramycin.

Malvisi et al. (1991) determined higher concentrations of oxytetracycline, neomycin and oleandomycin in milk of clinically diseased cows than in healthy cows, but the amount excreted via milk in percent of the total amount applied was lower in mastitic than in healthy cows for neomycin and oleandomycin.

Polymyxin B was not resorbed after intramammary treatment of healthy cows or cows with chronic mastitis. More than 90 % of the applied amount of drug was excreted via milk (Ziv and Schultze, 1982). Concentrations in healthy quarters were significantly higher than in acutely diseased quarters. In cows with experimentally induced *E. coli* mastitis only 55 % were excreted via milk within 24 hours. In these cases polymyxin B was also detected in serum and urine and in milk from untreated quarters. The excretion period was independent from the total amount of drug applied, but the excretion time was shorter in mastitic quarters than in healthy quarters.

### 2.2.3 Excretion from untreated quarters

The transfer from treated quarters to non-treated quarters has been described for a number of antibiotics. Transfer of penicillin to untreated quarters was already shown in 1964 by Edwards and was confirmed by later investigations in cows with or without clinical symptoms of mastitis (Rollins et al., 1970, Aureli et al., 1990). Rollins et al. (1970) also described the excretion of dihydrostreptomycin from untreated quarters. Freke and Bates (1981) detected low concentrations of penicillin and cloxacillin in milk from untreated quarters in clinically healthy cows.

Bräuning (1980) showed excretion from untreated quarters after treatment of healthy cows with drug combinations of penicillin+neomycin, penicillin+neomycin+oleandomycin and neomycin+tetracycline. In contrast, transfer of polymyxin B was only observed when cows with acute clinical mastitis were treated (Ziv and Schultze, 1982). Excretion of spiramycin from untreated quarters was dependent on treatment interval and dosage (Schällibaum et al., 1981).

For oleandomycin a higher transfer rate to untreated quarters was found compared to neomycin and oxytetracycline (Malvisi et al., 1991). Cefotaxime resp. ceftazidime were determined in milk of non-treated quarters already one hour after application (Rule et al., 1998).

### 2.2.4 Extra label use of antibiotics

Studies in extra label use of antibiotics are mainly related to dosages deviating from recommendations of the drug manufacturer.

Administering doses exceeding label directions were reported as one reason for inhibitor positive milk samples after the end of the withholding period in cows treated with different antibiotics for various reasons (Seymour et al., 1988).

A prolonged excretion period was observed in cows treated with a dosage of spiramycin twice as high as recommended by the manufacturer (Schällibaum et al., 1981).

Koppinen et al. (2001) determined factors influencing the excretion time of penicillin in milk in a field trial including 467 cows. In addition to the product used and the number of days in lactation also the total dosage applied had a significant influence on excretion time.

No exceeding of the withholding time for milk was observed after intramuscular application of ampicillin or amoxicillin resp. at higher dosages than recommended by the manufacturer (Anderson et al., 1996).

For certain antibiotics a combined intramammary and intramuscular therapy may increase the excretion time although Poelarends et al. (2001) did not find significant differences compared to only intramammary treatment in cows with clinical mastitis.

After intramammary treatment of cows with *Staphylococcus (S.) aureus* mastitis higher concentrations of ceftiofur in udder tissue were achieved by repeated intramammary infusions compared to single application (Owens et al., 1990). Highest concentrations of benzylpenicillin in untreated quarters were observed after repeated treatment in 24 hour intervals (Bjorland et al., 1998).

#### 2.2.5 Other factors influencing the excretion of residues in milk

The drug formula has a significant influence on the distribution within the udder and the excretion time in milk (Edwards, 1964, Moretain and Boisseau, 1989, Andrade et al., 1993, Koppinen et al., 2001)

In an investigation by Seymour et al. (1988) the antibiotic drug applied significantly influenced the excretion period in milk. Other factors like route of administration, case number, number of days treated, body weight, lactation number and milk yield had no significant influence on excretion time. The total dosage applied, the product used and the number of days in lactation had a significant influence on excretion time according to Koppinen et al. (2001). Moretain and Boisseau (1989) found the elimination of residues to be dependent on the factors quantity of antibiotic applied, nature of the vehicle and volume of the ointment infused for intramammary treatment.

Also after parenteral administration of antibiotic drugs residues are detected in milk. Cannon et al. (1962) found variation in penicillin excretion in milk between individual cows after intramuscular or intravenous administration, but no correlation could be established with milk production, fat content of milk or body weight.

In own investigations it could be shown that accidental storage of udder injectors at a too high temperature lead to significantly prolonged excretion periods in milk. This was probably due to changes in homogeneity and viscosity of the ointment (see D11).

### 2.3 Risk of bulk tank contamination

Microbial inhibitor tests routinely applied for testing of bulk tank milk have different sensitivities for the antibiotics applied in dairy cow treatment. In most countries screening tests with *Bacillus (B.) stearothermophilus* as test organism are applied for detection of inhibitors in routine monitoring. Not all antibiotics are detected at the Maximum Residue Level (MRL) fixed by Council Directive 2377/90. According to our own investigations only the beta-lactam antibiotics penicillin G, ampicillin and nafcillin were detected with sufficient sensitivity. The

detection limits of different test systems for the cephalosporine cefquinome were about 3.5 to 40 times the MRL concentration when applied as screening tests on incurred samples. For other antibiotics like dihydrostreptomycin and colistin the detection limits of *B. stearo-thermophilus* tests were about 100-10 000 times higher than the MRL concentration (D11).

Within an integrated detection system for antibiotic residues in milk additional group specific detection methods are applied. These tests have sufficient sensitivity to detect antibiotics which are not discovered at MRL by microbial inhibitor tests (Heeschen and Suhren, 1995).

In mastitis therapy beta-lactam antibiotics still play an important role. They are often applied as single substance or in combination with others to extend the therapeutic spectrum of a drug. It should be regarded that MRLs for some compounds of the beta-lactam group are extremely low (penicillin and ampicillin: 4 µg/kg, nafcillin: 30 µg/kg). Carry over of very small volumes of contaminated milk can lead to detectable concentrations of antibiotics in bulk tank milk.

A rough calculation shows the risk for exceeding the MRLs in bulk tank milk and thus for potentially inhibitor positive test results if carry over of milk from treated cows occurs (table 1). The highest risk is for penicillin because high concentrations occur in milk after udder treatment and the MRL for penicillin in milk is only 4 µg/kg. After treatment with the antibiotics listed in table 1 the accidental delivery of a milking from a treated cow would in all cases lead to a contamination of bulk tank milk with concentrations above the MRL.

<b>Table 1: Risk of contamination of milk at MRL concentration by carry over of milk from treated quarters in dependence of antibiotic substance</b>				
Antibiotic	MRL in milk [µg/kg]	Concentration in milk of treated quarter* [µg/kg]	Volume necessary to contaminate	
			10 liters of milk at next milking [ml]	1000 liters of bulk tank milk [ml]
Penicillin G	4	400 000	0.1	10
Ampicillin	4	100 000	0.4	40
Nafcillin	30	20 000	15	1500
Cefquinome	20	20 000	10	1000
Dihydrostreptomycin	200	30 000	67	6700
Colistin	50	30 000	17	1700

\* Highest concentration in cow composite milk after treatment of 4 quarters

These examples illustrate that prevention of residues in milk poses very high demands on automatic cleaning mechanisms applied after milking of treated cows.

### 3 Methodology and study materials

The investigations on excretion of antibiotic residues in cows with clinical mastitis were started on an experimental farm of the Research Institute for Animal Husbandry, Lelystad, NL. Sampling was performed by an automatic sampling device provided by Lely Industries, NL. 25 cows treated with antibiotics were sampled during treatment and withholding period. In addition, milk was sampled from those cows milked after the automated cleaning procedure following each milking of a treated cow to study the cleaning efficiency. During sample analysis performed by the Institute for Hygiene and Food Safety of the Federal Dairy Research Centre, Kiel, DE contamination of samples was noticed. Carry over between samples occurred because the tube filling sample vessels was not included into the short cleaning cycle. Although this does not affect milk quality no conclusions could be drawn from the results regarding excretion times in treated cows or cleaning efficiency.

Therefore additional experiments on cows with clinical mastitis were performed at the experimental farm Schaedtбек of the Federal Dairy Research Centre, Kiel, DE. Milking frequencies occurring in AM systems were simulated.

Experiments on cleaning efficiency of an AM system were carried out on an experimental farm of the Research Institute for Animal Husbandry, Lelystad, NL (chapter 3.5).

All milk samples were analysed for antibiotic residues in the Institute for Hygiene and Food Safety of the Federal Dairy Research Centre, Kiel, DE.

#### 3.1 Set up of treatment experiments

##### 3.1.1 Parameters for characterization of cows

Cows with naturally occurring clinical mastitis were used for the treatment trials. Cows were characterized by the following parameters:

- Case number per cow (not necessarily the same quarter)
- Number of lactation
- Days after calving
- Average milk yield per day during experimental period in kg
- Body weight in kg
- SCC of cow composite milk at milking time when mastitis was detected
- Severity of clinical symptoms: 1=flakes/slight clots, 2=clots, 3=gross abnormalities, 4=gross abnormalities and body temperature > 39.5°C
- Number of additional quarters with subclinical mastitis
- Bacterial pathogen isolated from the affected quarter
- Treatment success:
  - Bacteriological cure: pathogen not detected at the end of the experimental period and during two additional sampling times in weekly intervals
  - SCC of affected quarter < 100 000/ml at the end of the experimental period

### 3.1.2 Milking frequencies

For the treatment trials cows were milked with two different milking frequencies:

- 2 times per day at 7.00 a.m. and 5.00 p.m., milking intervals of 10 and 14 hours (reference group), 17 cases
- 1.5 times per day at 5.00 a.m. and 9.00 p.m. every second day and at 13.00 every other day, milking intervals of 16 hours, 5 cases

The milking time in which clinical signs of mastitis were detected was used as anamnesis.

### 3.1.3 Sampling

Sampling was performed during the experimental period consisting of anamnesis, treatment period, withholding period for milk plus 2 additional days.

From every milking during the experimental period the following samples were taken:

- Quarter milk samples (at the beginning of milking):
  - 10 ml without preservation for cyto-bacteriological investigation
- Cow composite milk:
  - 10 ml, preservation with potassium dichromate for determination of SCC
  - 250 ml, without preservation for determination of antibiotic residues

Samples were stored at 6 °C until analysis for 60 hours at maximum. Cow composite milk samples were stored at -20 °C for further investigations for 3 weeks at maximum. Samples for later re-examinations were preserved by lyophilization and stored at 6° C.

### 3.1.4 Drug

For treatment trials the following commercially available udder injector was used:

Drug:	Cobactan® LC
Manufacturer:	Hoechst Roussel Vet (now Intervet International), Unterschleissheim, DE
Composition:	88.8 mg Cefquinome(CEF)-sulfate = 75 mg CEF
Total volume per injector:	8 g
Withholding time for milk:	5 days
MRL for milk:	20 µg/kg
Dosage:	3 treatments within 24 hours, one injector per quarter per treatment was applied intracisternally into the affected quarter
Recommended treatment scheme:	3 treatments during successive milking times
Recommended storage:	below 30 °C
Total amount of pure substance applied per cow:	225 mg CEF

### 3.1.5 Treatment intervals

To apply the same quantity of antibiotic substance per time period the following treatment scheme was applied (table 2).

<b>Table 2: Treatment scheme for Cobactan® LC dependent on time of detection of clinical mastitis</b>				
<b>Milking frequency</b>				
	<b>2 times per day</b>		<b>1.5 times per day</b>	
<b>Detection of mastitis</b>	MT I*	MT II*	MT I*	MT II*
<b>Anamnesis</b>	day 1, 6.00 h	day 1, 16.00 h	day 1, 6.00 h	day 1, 16.30 h
<b>Treatment:</b>	day 1, 6.00 h	day 1, 16.00 h	day 1, 6.00 h	day 1, 16.30 h
<b>3 times per approximately 24 hours</b>	day 1, 17.00 h	day 2, 7.00 h	day 1, 14.30 h	day 2, 4.30 h
	day 2, 7.00 h	day 2, 17.00 h	day 2, 5.00 h	day 2, 14.00 h
<b>Further milking times</b>	7.00 h	17.00	21.00 h	5.00 h
	17.00 h	7.00 h	13.00 h	21.00 h
			5.00 h	13.00 h

\*MT I = morning milking, MT II = evening milking

## 3.2 Analytical methods

### 3.2.1 Udder health

SCC was determined according to IDF Standard 148A:1995, Method C, Fluoro-opto-electronic method.

The bacteriological investigation was performed according to the guidelines of the German Veterinary Association (DVG, 2000).

### 3.2.2 Quantitative detection of antibiotic residues - HPLC methods

For the identification and quantification of CEF a published HPLC-method was applied (Suhren and Knappstein, 2003). The method was described in detail in Deliverable D11.

## 3.3 Determination of withdrawal time

In order to compare the results of the experiments with different milking intervals the following approaches to determine the withdrawal period were used according to the approaches in healthy cows (D11).

### 3.3.1 Pragmatic approach

- Pragmatic - average

For each experiment the mean concentration ( $\mu\text{g}/\text{kg}$ ) of antibiotic in milk was calculated for every sampling time from the concentration of individual cows as determined by the HPLC-method. The sampling time after the last application was derived, where the average content

fell below the MRL of CEF (20 µg/kg) and did not exceed the MRL again. The mean was calculated from this time and the latest sampling time with an average value exceeding the MRL and defined as withdrawal time.

- Pragmatic - individual

The procedure described above was additionally applied for the individual cows of each experiment and the average withdrawal time calculated as mean from those values.

### 3.3.2 *Time-to Safe-Concentration (TTSC) method*

According to the Guidance of the European Agency for the Evaluation of Medicinal Products for the determination of withdrawal periods for milk (EMA, 1998) the TTSC-method calculates a tolerance limit for the number of milkings per animal. This tolerance limit is the time necessary for the residue concentration in milk of most animals to reach safe concentrations (i.e. the MRL). The method assumes a normal distribution after transformation of measured values onto the logarithmus naturalis (ln) scale. In order to derive the TTSC-points for each individual animal monotonic regression pre-processing of the data set was applied. Average and standard deviation of TTSC-points of the individual cows within experiment were calculated. According to the EMA-procedure the 95/95 tolerance limit was calculated by multiplication of the standard deviation with the indicated factor derived from the number of cows tested (e.g. in the case of 5 cows the factor is 4.210) and by addition of this product to the mean TTSC-value.

### 3.3.3 *Regression model*

Assuming a normal distribution after ln-transformation of measured concentrations quadratic as well as exponential regressions were calculated from the single concentrations and the time after last application within each experiment. From these regressions the intersections with the MRL-concentration and the upper limit of the 95 % confidence interval for an individual predicted value was computed.

## 3.4 **Analysis of variance**

In order to determine which factors have a systematic influence on the withdrawal time, an analysis of variance was carried out. For this purpose the GLM (General Linear Model) procedure of the statistic package SAS, release 8.01, was used.

The first time (in hours after the last application) when the concentration fell below the MRL was used as dependent variable (y). The following factors were included into the analysis: milking frequency per day (2 times, 1.5 times), days after calving ( $\leq 60$  d,  $>60$  d) and number of lactation (1,  $>1$ ). Average milk yield per day, SCC in cow composite milk at milking time

of onset of disease (Log<sub>10</sub> transformation) and body weight as continuous variables were used as covariate.

The linear model had the following equation:

$$Y_{ijkl} = \mu + mf_i + dac_j + ln_k + b_1(X_{ijkl}) + b_2(X_{ijkl}) + b_3(X_{ijkl}) + e_{ijkl}$$

With:

$Y_{ijkl}$  = dependent variable (first time (h) when the antibiotic content fell below the MRL)

$\mu$  = overall mean

$mf_i$  = effect of the  $i^{\text{th}}$  milking frequency

$dac_j$  = effect of the  $j^{\text{th}}$  days after calving

$ln_k$  = effect of the  $k^{\text{th}}$  lactation number

$b_1$  = slope for milk yield

$b_2$  = slope for SCC

$b_3$  = slope for body weight

$e_{ijkl}$  = random residual error

The calculation was also performed without the factor body weight. In addition, the following factors were used as additional factors in the model for the group milked two times per day instead of milking frequency: Severity of disease ( $\leq 2$ ,  $> 2$ ), number of additional quarters affected by subclinical mastitis (0, 1, 2, 3) and treatment success (bacteriological cure - yes/no and SCC  $< 100000/\text{ml}$  at the end of the withholding period - yes/no, respectively)

### 3.5 Efficiency of short cleaning cycle to prevent carry over of antibiotic residues

In addition, on one of the experimental farms of the Research Institute for Animal Husbandry Lelystad, NL an experiment was performed to determine the efficiency of the short cleaning cycle in an AM system after milking of treated cows. In an AM system milk samples were taken from the milk of three cows which had previously received Cobactan<sup>®</sup> (compound CEF) treatment. After those cows a unit flush with 10-12 l cold water was performed. The milk of cows milked directly after the unit flush was sampled as well.

Samples were analysed by the Federal Dairy Research Centre, Kiel, DE. The screening test Delvo SP (DSM Food Specialities, Dortmund, DE) was applied for detection of CEF residues in milk samples of treated cows. The detection limit of this test for CEF is 70  $\mu\text{g}/\text{kg}$  in incurred samples. Undiluted samples and samples after 1:10 and 1:100 dilution were tested. For detection of residues in milk samples from cows milked after treated cows the Beta Star test (Chr. Hansen GmbH, Nienburg, DE) with a detection limit of 10  $\mu\text{g}/\text{kg}$  for CEF in incurred samples was applied (D11).

## 4 Results

### 4.1 Status of animals

The status of the animals included in the experiments is summarized in table 3.

<b>Table 3: Status of cows from treatment trials in cows with clinical mastitis</b>											
Cow No. (Case No.)	No. of lactation	Days after calving	Milk yield per day [kg]	Body weight [kg]	Treated quarter(s)	SCC in composite milk [1000/ml]	Severity of disease	No. of addi- tionally af- fected quar- ters	Mastitis pathogens	Bacteriolog. cure	SCC <100 000/ml
<b>Milking frequency 2 times per day</b>											
1808 (1)	3	206	23.1	790	LF	666	3	3	CNS	Yes	No
1808 (2)	3	246	23.5	792	LF	2660	1	3	<i>S. aureus</i>	No	No
1818	4	36	37.0	723	LF	1021	3	0	<i>Sc. uberis</i>	Yes	Yes
1825	2	252	22.3	770	RF	2002	3	3	Coryneforms	No	No
1829	3	51	37.9	720	LH	1707	1	0	no growth	-	Yes
1846	2	422	10.3	732	RH	n.a.	4	1	Coryneforms	No	No
1869 (1)	2	54	35.4	660	RH	1917	3	0	<i>Sc. uberis</i>	No	No
1869 (2)*	2	69	31.6	655	LF	6976	4	2	<i>Sc. uberis</i>	No	No
1869 (3)	2	99	34.2	670	LF	1085	3	1	<i>Sc. uberis</i>	Yes	Yes
1878 (1)	2	12	27.1	670	RF	3802	3	1	<i>Sc. uberis</i>	No	No
1878 (2)	2	37	21.0	670	RH	1255	2	2	<i>E. coli</i>	Yes	No
1890	1	17	16.9	n. a.	LH	2659	3	3	Enterococci	No	No
1891	1	71	22.0	704	LH	3380	3	0	<i>E. coli</i>	Yes	No
1900	1	3	25.2	610	RH, LH	470	3	2	CNS/CNS	Yes/Yes	Yes/Yes
1907 (1)	1	98	26.3	626	LH	1143	3	0	<i>S. aureus</i>	No	No
1915 (1)	1	2	21.2	625	LF	718	1	2	<i>E. coli</i>	Yes	No
1915 (2)	1	40	26.3	623	RH	322	1	0	no growth	-	Yes
<b>Milking frequency 1.5 times per day</b>											
1729	5	1	24.0	842	RH	1707	2	2	Enterococci	Yes	No
1815	4	36	35.6	851	RF, RH	1419	1	2	<i>Sc. uberis</i> + Coli- forms/Coliforms	Yes/Yes	No/Yes
1868 (1)	2	56	14.6	768	LF	3817	2	2	<i>E. coli</i>	Yes	No
1868 (2)	2	90	15.5	702	RH	6062	1	1	<i>Sc. uberis</i> + CNS	No	Yes
1907 (2)	1	147	21.5	620	LF	1653	3	3	no growth	-	Yes

\* At the second case of mastitis cow no. 1869 received 650 mg CEF i.m. with the third intramammary treatment

## 4.2 Udder health

In figures 1 to 3 some examples on the development of SCC in quarters and cow composite milk after treatment are shown. In cow 1818 (figure 1) treatment was successful and SCC decreased rapidly in the affected quarter and thus in cow composite milk. At the end of the withholding period for Cobactan<sup>®</sup> LC of 120 hours the SCC was below 100 000/ml.

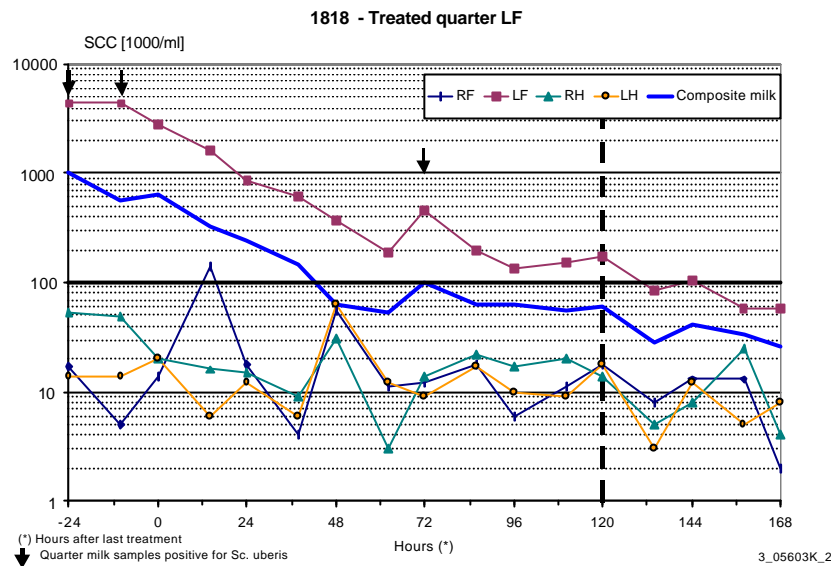


Figure 1: Development of SCC in quarter milk samples and cow composite milk after treatment - cow no 1818 - mastitis caused by *Sc. uberis* - success of treatment

In cow 1869 (1) the initial drop in SCC may be interpreted as treatment success although the quarter had not been cleared of the pathogen. 48 hours after the last treatment *Sc. uberis* was detected again. Another increase in SCC in the affected quarter followed which caused an increase of SCC in cow composite milk (figure 2).

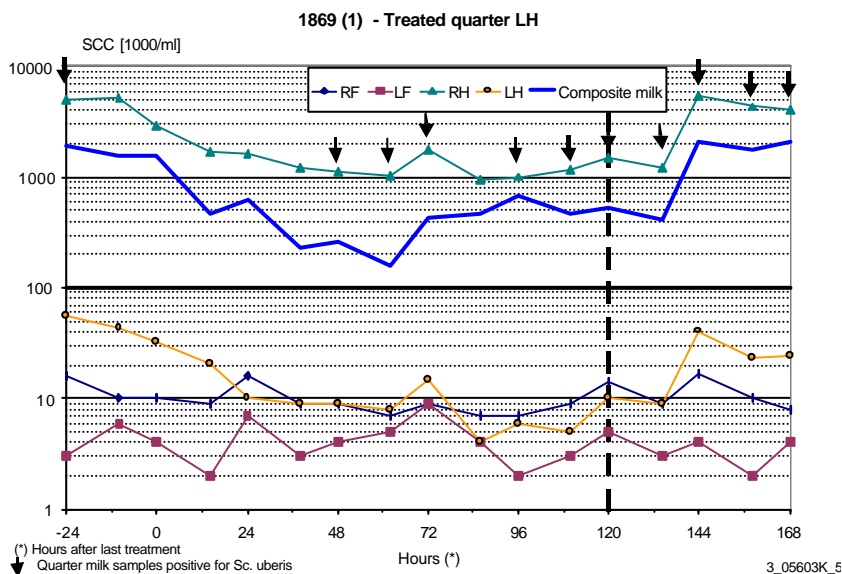


Figure 2: Development of SCC in quarter milk samples and cow composite milk after treatment - cow no. 1869 - mastitis caused by *Sc. uberis* - no success of treatment

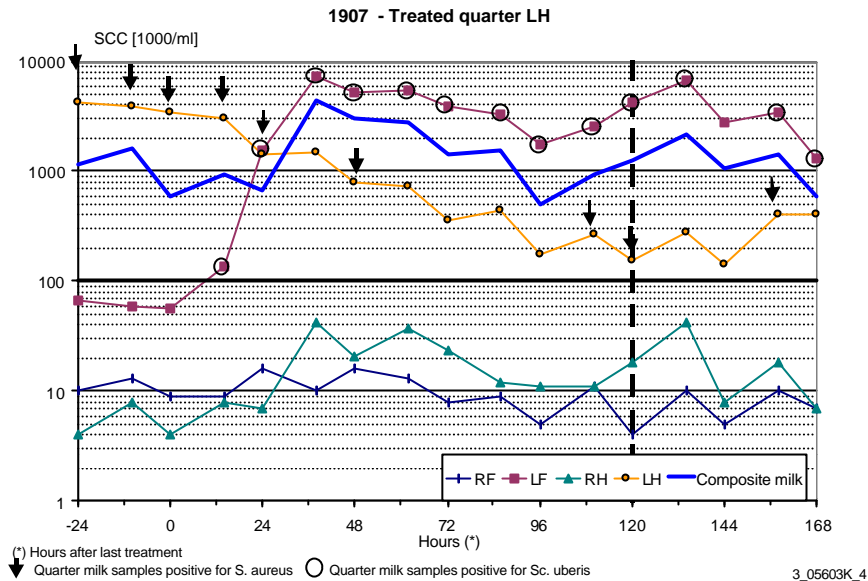


Figure 3: Development of SCC in quarter milk samples and cow composite milk after treatment, cow no. 1907, mastitis caused by *S. aureus* - no success of treatment, new infection of LF quarter by *Sc. uberis*

In cow 1907 treatment was not successful (figure 3). Although SCC of the affected quarter decreased, intermitting excretion of *S. aureus* could still be seen after end of treatment. In addition, a new infection of another quarter occurred during the experimental period and lead to a high increase of SCC in cow composite milk.

Bacteriological cure of intramammary infections after treatment was only seen in 12 of 21 quarters (57 %). In milk of three quarters no bacterial pathogen was detected when clinical symptoms of mastitis were observed. None of the quarters infected with *S. aureus* (n=2) or coryneforms (n=2) was cleared of the pathogen, whereas all quarters with infections by coagulase negative staphylococci (n=3) or *E. coli* (n=4) were bacteriologically negative at the end of the experimental period. 2 of 5 *Sc. uberis* infections and one of 2 infections with enterococci as well as one of two mixed infection were cured (table 3).

### 4.3 Excretion of residues in milk

In table 4 the quantity of CEF excreted via milk is summarized per cow and per group in dependence of the milking interval.

The amount of antibiotic residues excreted via milk in percent of the total amount applied varied largely between individual cows.

For the group milked 1.5 times per day the total percentage excreted via milk was on average lower (18.0 %) than for the group milked 2 times per day (26.7 %).

In both groups the percentage excreted in milk was lower than in clinically healthy cows milked in the same intervals with 43.1 % for cows milked 2 times per day and 51.5 % for cows milk 1.5 times per day (D11).

Interesting is the very low percentage of excretion via milk in the two cows which had severe mastitis with fever: In cow no. 1846 only 4.4 % of the applied dosage was excreted via milk. A similar result was found in cow no. 1869 during the second case of mastitis. This cow received an additional intramuscular treatment with Cobactan® due to fever above 40 °C on the second day of disease. In this case results are not fully comparable because of the different route of administration.

<b>Table 4: CEF excretion via milk in dependence of milking interval in cows with clinical mastitis</b>			
<b>Cow no.</b>	<b>Milk yield [kg]</b>	<b>Excreted CEF</b>	
		<b>in mg</b>	<b>in % of applied*</b>
<b>Milking frequency: 2 times per day</b>			
1808 (1)	23.1	71	31.6
1808 (2)	23.5	32	14.2
1818	37.0	105	46.7
1825	22.3	86	38.2
1829	37.9	47	20.9
1846	10.3	10	4.4
1869 (1)	35.4	44	19.6
1869 (2)*	(31.6)	(43)	(4.9)
1869 (3)	34.2	101	44.9
1878 (1)	27.1	44	19.6
1878 (2)**	(21.0)	-	-
1890	16.9	102	45.3
1891	22.0	47	20.9
1900***	(25.2)	(135)	(30.0)
1907 (1)	26.3	35	15.6
1915 (1)	21.2	55	24.4
1915 (2)	26.3	62	27.6
<b>Average</b>	<b>26.0</b>	<b>60</b>	<b>26.7</b>
<b>Milking frequency: 1.5 times per day</b>			
1729	24.6	39	17.3
1815***	(35.6)	(169)	(37.6)
1868 (1)	14.6	33	14.7
1868 (2)	15.5	25	11.1
1907 (2)	21.5	65	28.9
<b>Average</b>	<b>19.1</b>	<b>41</b>	<b>18.0</b>
* additional treatment with 650 mg CEF i.m. at 3rd intramammary treatment due to fever			
** one sample missing			
*** treatment of 2 quarters, % excretion calculated from total amount of 450 mg CEF applied			
*, ** and *** were excluded from calculations of averages			

Highest total amounts excreted via milk were determined in those cows which were treated on two quarters (1900 and 1815). For these cows also the highest concentrations of antibiotic residues in cow composite milk were observed (figure 4 and 5).

#### 4.4 Withdrawal time

The excretion curves of the individual cows within each experiment are demonstrated in figures 4 and 5.

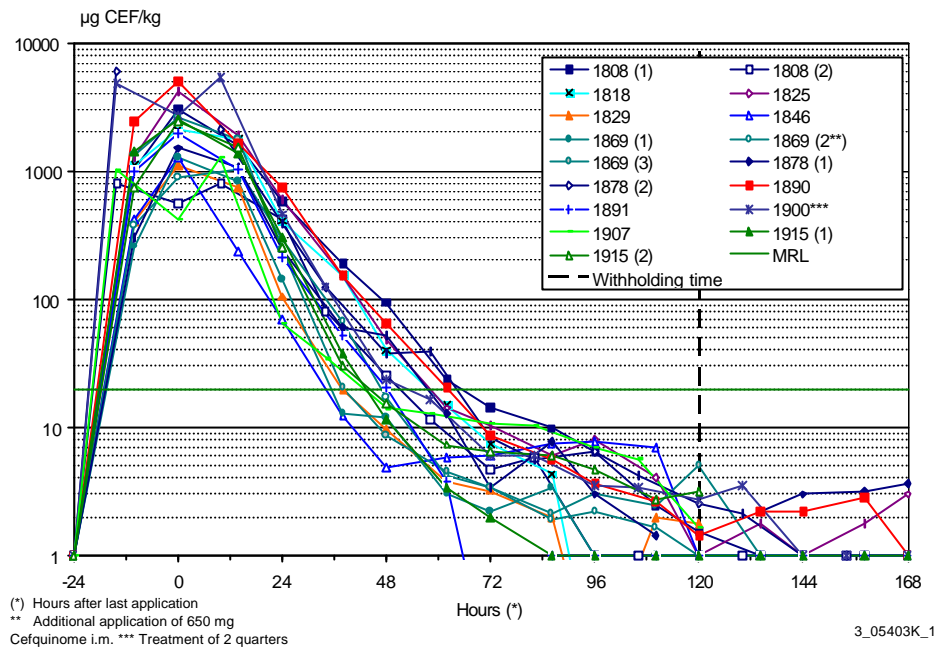


Figure 4: Excretion of cefquinome (Cobactan® LC) in milk of cows with clinical mastitis, milking frequency: 2 times per day

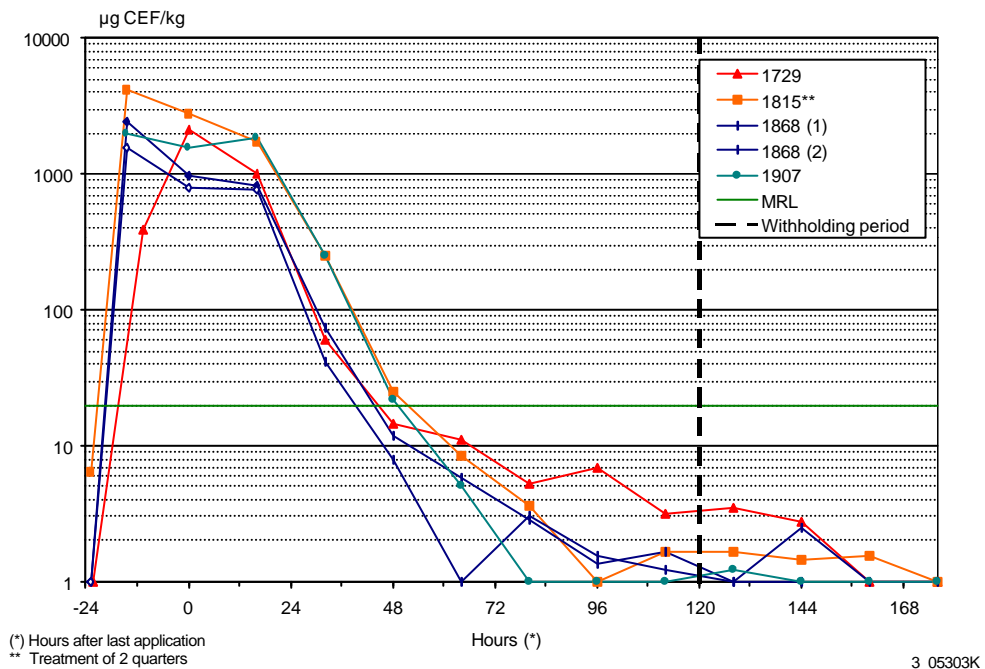


Figure 5: Excretion of cefquinome (Cobactan® LC) in milk of cows with clinical mastitis, milking frequency: 1.5 times per day

In both groups large variation between the excretion patterns of individual cows was observed. In all cases the concentrations in milk were below the MRL at 72 hours after the last

treatment. In no case concentrations exceeding the MRL were detected after the end of the withholding period of 120 hours.

A tendency of shorter excretion periods in those cows milked with longer intervals was observed. In figures 6 and 7 the different excretion curves are shown for cows milked 2 times per day in dependence of time of first treatment.

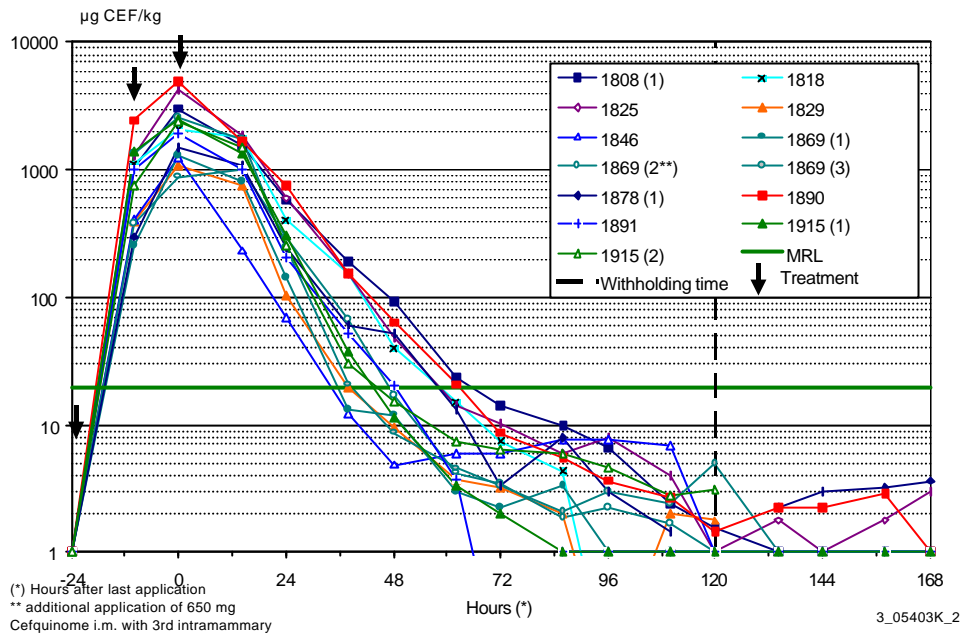


Figure 6: Excretion of cefquinome (Cobactan® LC) in milk of cows with clinical mastitis, milking frequency: 2 times per day; first treatment in evening milking time - 13 cows

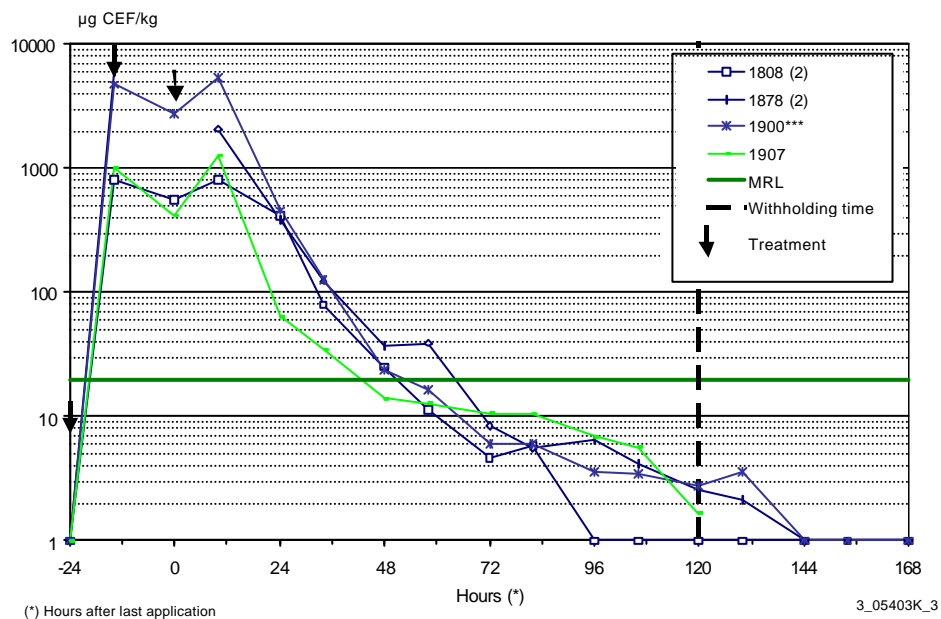


Figure 7: Excretion of cefquinome (Cobactan® LC) in milk of cows with clinical mastitis, milking frequency: 2 times per day; first treatment in morning milking time - 4 cows

From the form of the curves in the three milkings after intramammary treatment it can be seen that the concentration in milk is dependent on the time interval between last treatment and milking. When cows were milked 14 hours after the last intramammary application the concentration in milk was lower than when milking was already after 10 hours (figure 6 and 7).

The average excretion curves of the different treatment groups are shown in figure 8.

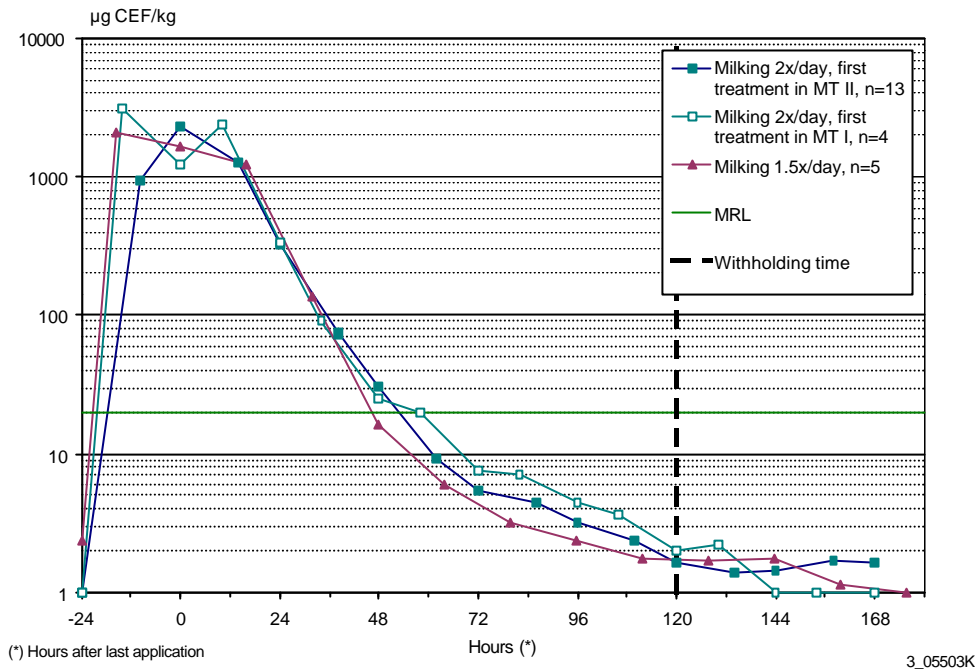


Figure 8: Excretion of cefquinome (Cobactan® LC) in milk of cows with clinical mastitis, averages of different treatment groups

When the same cows were treated repeatedly the excretion curves were sometimes deviating from each other whereas in some cases they were very similar (Figures 9 to 14).

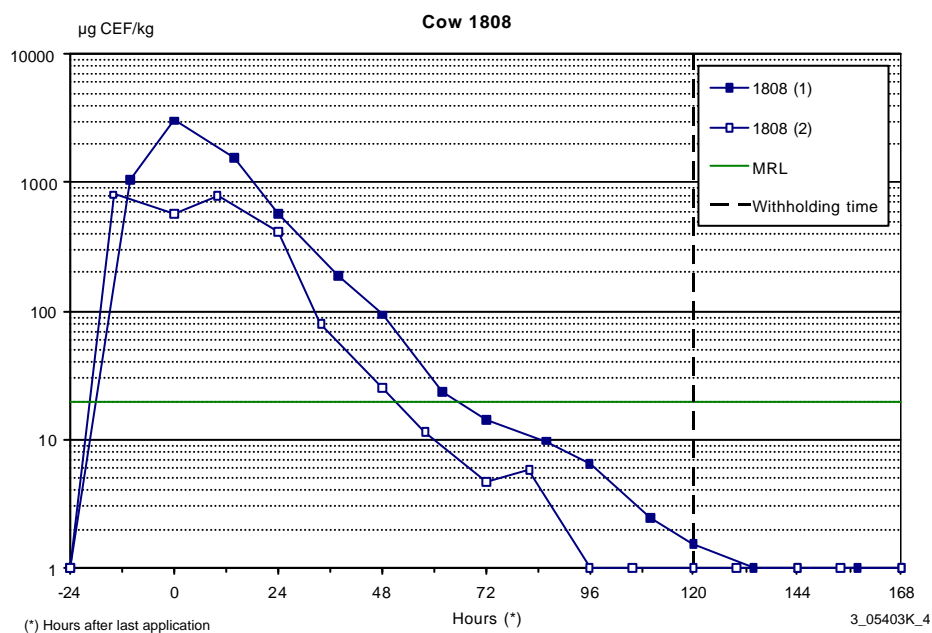


Figure 9: Excretion of cefquinome (Cobactan® LC) in milk of cows with clinical mastitis, milking frequency: 2 times per day; cow 1808

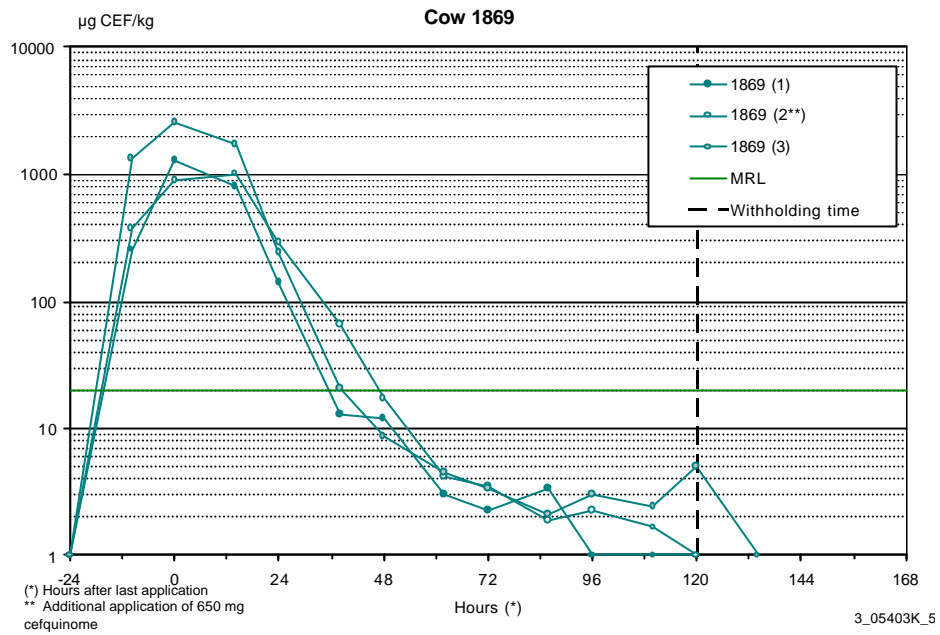


Figure 10: Excretion of cefquinome (Cobactan<sup>®</sup> LC) in milk of cows with clinical mastitis, milking frequency: 2 times per day; cow 1869

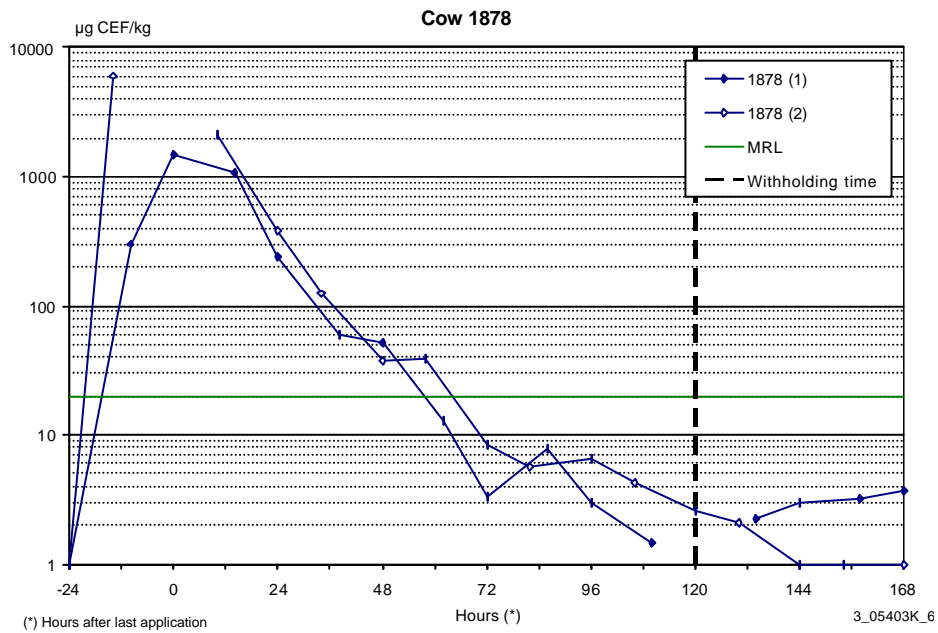


Figure 11: Excretion of cefquinome (Cobactan<sup>®</sup> LC) in milk of cows with clinical mastitis, milking frequency: 2 times per day; cow 1878

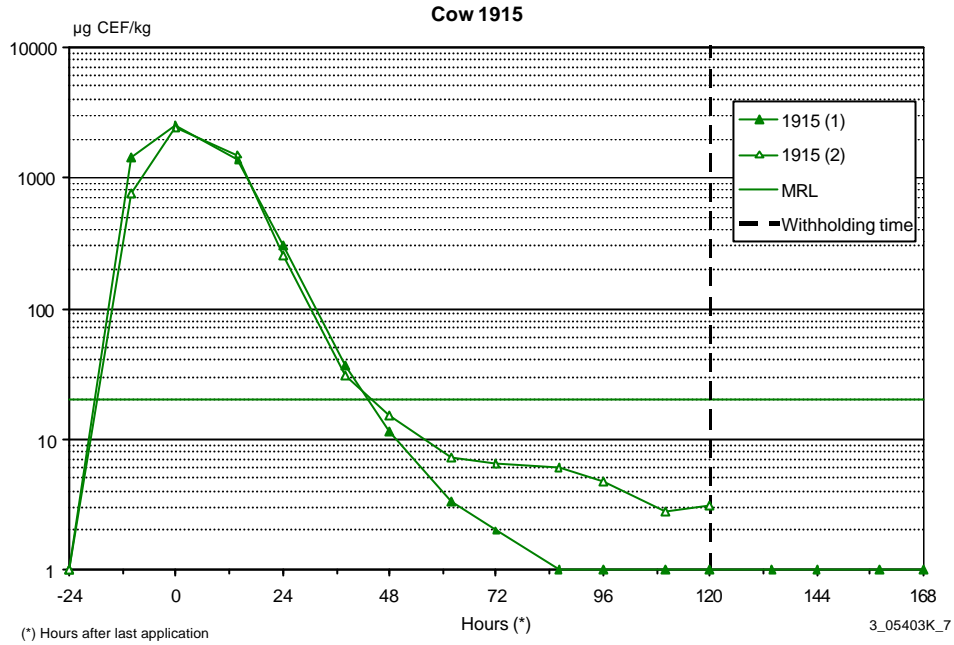


Figure 12: Excretion of cefquinome (Cobactan<sup>®</sup> LC) in milk of cows with clinical mastitis, milking frequency: 2 times per day; cow 1915

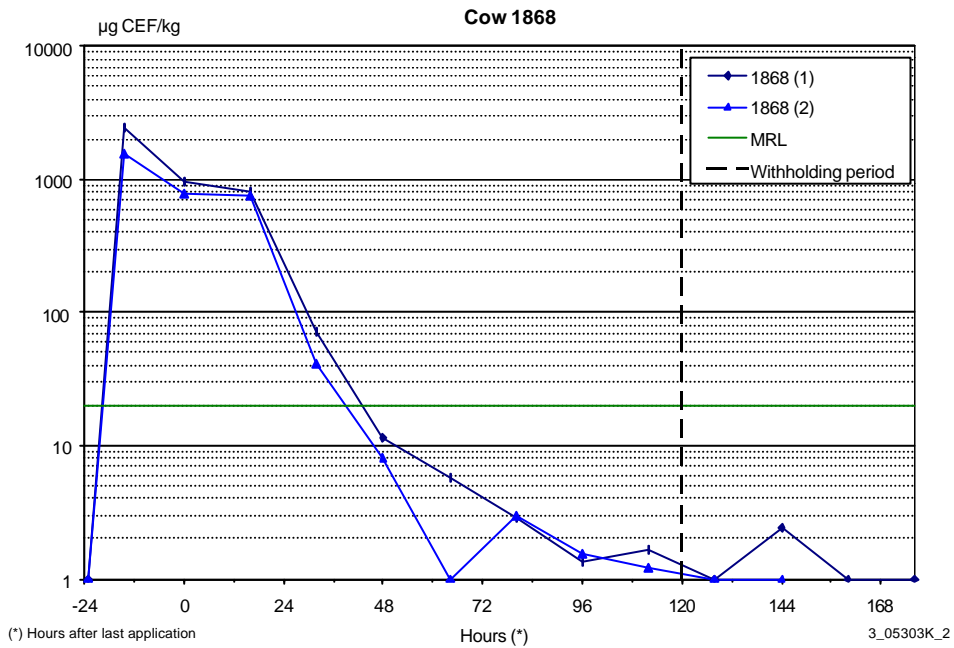


Figure 13: Excretion of cefquinome (Cobactan<sup>®</sup> LC) in milk of cows with clinical mastitis, milking frequency: 1.5 times per day; cow 1868

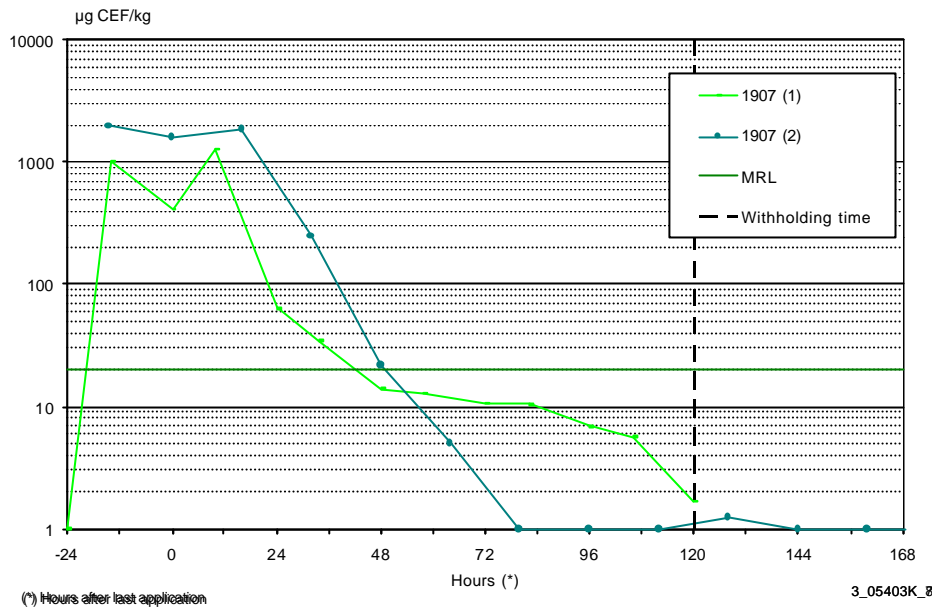


Figure 14: Excretion of cefquinome (Cobactan<sup>®</sup> LC) in milk of cows with clinical mastitis, milking frequency: 2 times per day (1) resp. 1.5 times per day (2); cow 1907

The quadratic and exponential regressions for the treatment groups milked 2 times and 1.5 times per day resp. are shown in figure 15.

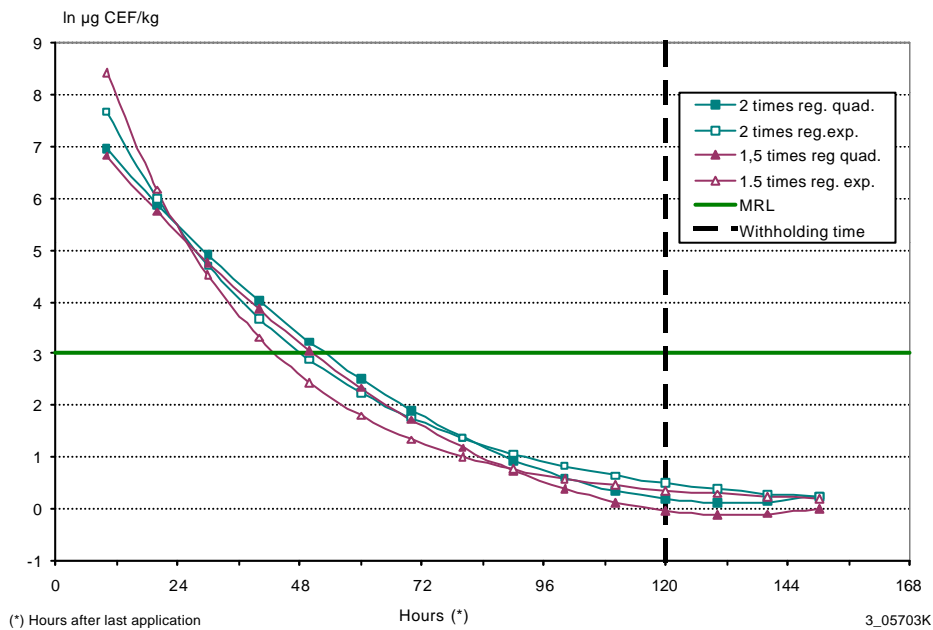


Figure 15: Excretion of cefquinome (Cobactan<sup>®</sup> LC) in milk of cows with clinical mastitis in dependence of milking frequency - quadratic and exponential regressions

The results of the withdrawal times derived by different approaches are summarized in table 5. Data from those cows which received treatment on 2 quarters or additional intramuscular treatment were excluded from the analysis.

<b>Table 5: Withdrawal time of CEF (in hours) in dependence of milking frequency in cows with clinical mastitis – summarized results from pragmatic approach, TTSC and regression models; Official withdrawal time: 120 h</b>		
<b>Approach</b>	<b>Mean</b>	<b>95/95 tolerance limit/ 95 % confidence interval</b>
<b>Milking frequency : 2 times per day</b>		
Pragmatic – individual	49	
Pragmatic – average	56	
TTSC <sup>a</sup>	54	66
Quadratic regression <sup>b</sup>	53	81
Expon. regression <sup>b</sup>	48	79
<b>Milking frequency : 1.5 times per day</b>		
Pragmatic – individual	44	
Pragmatic – average	40	
TTSC <sup>a</sup>	52	108
Quadratic regression <sup>b</sup>	51	75
Expon. regression <sup>b</sup>	43	60
<sup>a</sup> 95/95 tolerance limit, <sup>b</sup> 95 % confidence interval		

It has to be regarded, that only data from 4 treatments were included in the group milked 1.5 times per day. Therefore a high factor for calculations of the 95/95 tolerance limit had to be applied in the TTSC method. For the group milked 2 times per day the tolerance limit is smaller because of a higher number of cases (n=13).

#### 4.5 Analysis of variance

For the analysis of variance the same model was applied as in healthy cows (Chapter 3.4 and D11). Data from those cows which received treatment on 2 quarters or additional intramuscular treatment were excluded from the analysis.

Without the factor body weight the model accounted for 35 % of deviation in excretion time. Of the included factors only milk yield was of significant influence on excretion time with shorter excretion times in cows with higher milk yield ( $p < 0.05$ ). The predicted values for the withdrawal time in the two groups are summarized in table 6.

<b>Table 6: Withdrawal time (in hours) for CEF (Least Square Means (LSQ<sub>M</sub>) and standard error)</b>		
<b>Milking frequency</b>	<b>LSQ<sub>M</sub> ± se</b>	<b>sign. *</b>
2 times	57.7 ± 3.0	a
1.5 times	45.5 ± 6.4	a
* Different letters show significant differences ( $p < 0.05$ )		

By including the factor body weight 34 % of variance were explained by the model, but none of the factors had a significant influence on excretion time.

Other factors like case number within cow, severity of disease or treatment success did not have a significant influence on excretion time and were therefore not included in the model.

#### 4.6 Efficiency of short cleaning cycle to prevent carry over of antibiotic residues

The results from the experiment on carry over of CEF residues for milk of treated cows is shown in table 7.

<b>Table 7: Results of screening tests on milk samples from treated cows and subsequently milked cows</b>				
<b>Cow-No</b>	<b>Delvo SP*</b>			<b>Beta Star*</b>
	<b>undiluted milk</b>	<b>1:10</b>	<b>1:100</b>	<b>undiluted</b>
1352	4	3	1	
2249	1			1
1937	4	3	1	
2248	1			1
1779	4	3	1	
2284	1			1

\* 4 = positive, 3 = suspicious, 1 = negative;  treated cows

From the results of the Delvo SP test it can be concluded that the concentration of CEF in milk samples of treated cows was about 700 µg/kg, because the detection limit for CEF in incurred samples is about 70 µg/kg (D11). No residues were detected by application of Delvo SP and Beta Star test in the milk of cows milked directly after the short cleaning cycle following milking of treated cows. The detection limit of the Beta Star test for CEF is about 10 µg/kg in incurred samples and thus far below the MRL.

## 5 Discussion

For experiments on excretion of antibiotic residues in cows with clinical mastitis milked with different milking frequencies Cobactan<sup>®</sup> LC was selected because large variation was observed in excretion times for this drug in healthy cows. In worst case experiments concentrations of cefquinome in milk above the MRL of 20 µg/kg were detected shortly before the end of the withholding period and in one cow still at 120 hours after the last treatment (D11).

### 5.1 Udder health status

Large differences regarding severity of disease were observed in cows with clinical mastitis included in the treatment trials ranging from slight clots/flakes in foremilk samples to severe mastitis with gross abnormalities of milk and fever. 6 different bacterial species as well as combinations were isolated from affected quarters. Severity of disease was not related to the pathogen involved.

The results from characterization of cows clearly show that large variation occurred regarding the individual factors which may influence the excretion time of residues in milk. These findings explain why withholding periods are usually determined by excretion studies in healthy cows to ensure comparability between cows.

Bacteriological cure was only achieved in 12 of 21 bacteriological positive quarters. Comparisons between cows milked with different milking intervals regarding the cure rate are not possible because of the low number of cows included in the group milked 1.5 times per day. Also the different spectrum of mastitis pathogens involved as well as repeated treatment of the same quarter in 2 cows make comparisons difficult. Even in the same cows different duration of excretion periods of residues in milk were observed when treatment was repeated within the same cow at the same or another udder quarter.

Before delivering milk to the bulk tank after the end of the withholding period the visual appearance and SCC of milk of treated cows should be checked, e.g. by California Mastitis Test, because absence of clinical symptoms is no guarantee for bacteriological cure and SCC can still be very high if the mastitis pathogens persist in infected quarters (Figures 2 and 3).

### 5.2 Influence of milking frequency

In all cows with clinical mastitis the concentration of cefquinome in milk was below the MRL at the end of the withholding time. In case of Cobactan<sup>®</sup> LC decreased milking frequency in cows with mastitis did not increase the risk for violation of the withholding time. No significant differences were determined regarding the length of excretion periods in cows milked two times per day compared to cows milked 1.5 times per day.

The excretion time in milk was much shorter than in experiments performed in healthy cows with the same milking frequencies ranging from 97 to 109 hours for cows milked 2 times per day and from 96 to 104 hours for cows milked 1.5 times per day (D11). Even by including the

95/95 tolerance limit resp. the 95 % confidence interval the withholding time of 120 hours was sufficient in cows with clinical mastitis.

In interpreting these results it has to be considered that the experiments in healthy cows were performed as worst case trials with treatment of all 4 quarters at three treatments. So the total amount of drug applied was 4 times as high as applied in most of the clinical cases. Only in 2 diseased cows 2 quarters were treated simultaneously whereas the others received treatment of only one quarter. Thus the longer excretion periods in healthy cows may have been caused by the higher dosage applied.

In cows with mastitis the concentration in milk was also influenced by the interval between treatment and next milking. Cows milked 1.5 times per day had in general lower concentrations in milk than cows milked 2 times per day. The shorter the milking interval after treatment the higher was the concentration found in milk. This could also be seen in cows milked two times per day, because there were slight differences dependent on the time of milking after treatments (10 and 14 hours resp.) (Figures 6 and 7).

By different methods for calculation of withholding times no obvious differences between the two groups milked with different milking frequencies were detected. When interpreting the results it has to be regarded that the group milked 1.5 times per day consisted of only 4 cows. Therefore a high factor had to be applied for the calculations according to the TTSC method. Thus the shorter withholding time in cows milked 2 times per day when the 95/95 tolerance limit is included is only based on the different number of cows in the experiments.

Also due to the results from the analysis of variance the milking frequency had no significant influence on the excretion time in milk. This is in accordance with the observation in healthy cows where no significant differences were determined between cows milked 2 times per day resp. 1.5 times per day for Cobactan<sup>®</sup> LC (D11). The only factor which had a significant influence on the excretion time was the average milk yield per day with shorter excretion times in cows with higher milk yield. This influence factor was no longer significant when body weight was included into the analysis.

It should be emphasized that the results from our study can not necessarily be transferred to other drugs. Several studies have been conducted on excretion of drugs in milk of cows with clinical mastitis. Although for some drugs shorter excretion periods were found in mastitic cows after intramammary treatment, e.g. for macrolides and polymyxin B (Nouws and Ziv, 1979, Schällibaum, 1981, Ziv and Schultze, 1982) for others like penicillin and cefacetrile the excretion was found to be prolonged (Edwards, 1964, Schluep et al., 1979). After intramuscular or intravenous treatment higher concentrations and longer excretion may be found in milk of cows with mastitis (Erskine et al., 1995). It has to be regarded that the drug basis has also an important influence on the excretion time and may influence the behaviour in diseased cows in different ways (Schluep et al., 1979). Different excretion times have also been found for chronic and acute mastitis (Ziv and Schultze, 1982). So shorter or prolonged milking intervals may have different consequences in dependence of the actual status of disease in individual cows.

Although in this study no prolonged excretion times were found for cows milked with longer intervals experiments in healthy cows in general revealed longer excretion times in cows milked less frequently compared to cows milked 3 times per day for several antibiotics (D11). The setting of withholding periods is based on milking frequencies of two times per day. Therefore this should also be the minimum milking frequency for treated cows milked in AM systems. Checking the success of treatment two times per day is also recommended to control the development of disease at least in the early stages after treatment.

### **5.3 Total amount of drug excreted via milk**

The amount of CEF excreted in milk as percent of the total amount of drug applied was lower in cows with mastitis (18.0 and 28.7 % resp.) than in healthy cows (51.5 and 43.1 % resp.) for milking frequencies of 1.5 times or 2 times per day. This was in contrast to the expectations derived from the pharmacokinetic properties of CEF. Because CEF is a weak acid higher milk to serum concentrations had been expected in cows with clinical mastitis compared to healthy cows, because the pH of mastitic milk is usually higher than in milk of healthy cows (Ziv 1975). This would have lead to higher amounts excreted via milk. Probably in case of CEF the low lipid solubility is of higher importance for the excretion via milk. Thus the drug may only be able to penetrate the blood milk barrier in case of damage due to mastitis. This would explain the lower amount of CEF excretion via milk compared to healthy cows. Similar findings have also been presented for polymyxin B, another antibiotic with low lipid solubility. For polymyxin B the total amount excreted via milk was reduced from 90 % in healthy cows to 55 % in cows with acute mastitis (Ziv and Schultze, 1982).

Interesting is that in cows with severe clinical symptoms of mastitis and fever the total amount excreted via milk was less than 5 % (table 4). Fever may be interpreted as an indication for severe damage of the blood milk barrier.

The penetration of the blood milk barrier would also explain the lower CEF concentrations in milk with longer intervals between treatment and milking. Unfortunately no experiments have been performed with cows milked more often than two times per day, because from the results in healthy cows mainly prolonged milking intervals seemed to increase the risk for exceeding the withholding time. Although higher concentrations have to be expected in milk of cows milked in shorter intervals, it is not expected that the withholding time is exceeded when one or 2 quarters are treated per cow.

### **5.4 Dosage of applied drug**

Results from earlier studies during this workpackage (D11) showed that deviations from treatment schemes recommended by the drug manufacturer can lead to variable concentrations of antibiotics and deviating excretion times in milk. Also in this trial it can be seen, that cows treated on two quarters have higher concentrations of CEF in milk than cows with only one treated quarter (figures 4 and 5). Additional intramuscular treatment may also increase the concentration in milk in cows with clinical mastitis. This can be seen in the results from cow

1869 (figure 10), which received intramuscular treatment on the second day of disease during the second case of mastitis. This led to a higher concentration in milk at 14 hours after the last treatment than would have been expected from the curve form derived from two other cases in the same cow. Also the longest excretion time was observed in this cow when the drug was administered i.m. in addition to intramammary treatment. But this is not necessarily due to the treatment scheme, because large variation has also been observed in other cows receiving the same treatment during two mastitis cases.

Prolonged excretion of residues in milk after extra label use of antibiotics with higher dosages than recommended by the manufacturer have been reported for different antibiotics (Schällibaum, 1981, Seymour et al., 1988, Koppinen et al., 2001). Therefore deviating excretion times have to be regarded when cows in AM systems are treated and milked in unusual intervals.

Milk from untreated quarters must not be delivered because for several groups of antibiotics transfer from treated to untreated quarters has been reported (Chapter 2.2).

### **5.5 Efficiency of short cleaning cycle to prevent carry over of antibiotic residues**

Due to our results carry over of residues into milk of untreated cows did not occur when a short cleaning cycle was performed in an AM system after milking of treated cows. The results from this experiment should be interpreted with care because the number of samples was limited and only one antibiotic and one AM system were studied. The experiments have been performed with CEF. Due to our results higher concentrations of CEF than 700 µg/kg may be expected in milk of treated cows in the first milking after treatment. In addition, the volume of milk from penicillin-treated cows that is necessary to contaminate milk from untreated cows with concentrations above the MRL level is smaller than for CEF (table 1).

According to a study by Rasmussen (2003) the percentage of inhibitor positive bulk tank samples from farms with AM systems was 0.48 % in 2002, but the volume necessary to contaminate bulk milk to cause inhibitor positive tests is about 100 times higher than to contaminate one milking of an untreated cow. It can not be excluded that in practice carry over of residues occurs more frequently to the milk of the next milking without detection in bulk milk. Pallas (2002) found 27 % of inhibitor positive samples when individual cows milked after treated cows were sampled in an AM system, although it was not clear if the technical function of the cleaning system had been checked prior to the study. The antibiotics used for treatment were not identified, so other sources of inhibitors like disinfectants can not be excluded as causes of positive inhibitor tests. No information was given on the cleaning process itself in this study.

Manufacturers of AM systems as well as farmers should be aware of the fact that only small volumes of milk containing antibiotics are necessary to contaminate bulk tank milk with concentrations exceeding the MRL. The proper function of the system for automatic diversion of milk for treated cows and for cleaning of milk contact surfaces is essential to prevent carry over of antibiotic residues.

## 5 Conclusions

In the following recommendations are made for the management of cows in AM systems treated with antibiotics to prevent contamination of bulk tank milk. The recommendations are based on our findings on excretion of antibiotic residues in healthy and diseased cows milked with different milking frequencies (D11 and this report) complemented by information from the available literature. Several of the recommendations are not specific for automatic milking but are also valid for conventional milking.

### Recommendations for the farmer

- The farmer should ensure that milk from treated animals is not delivered to the bulk tank. To avoid accidental milking of treated cows treatments should be reported to the AM system before administering a drug if cows are not separated from the herd. If treatment by a veterinarian is expected cows should be kept separate after treatment until the information on treatment is filed into the management system of the AM system. This is essential when treatment by the veterinarian is performed in absence of the farmer. Alternatively animals could be identified as treated in advance. An agreement on the appropriate procedure should be sought with the veterinarian.
- Good records should be kept on treatment of cows in order to determine appropriate withholding periods. An update of the information may be necessary if cows are treated repeatedly. This is also important for drying off treatment with long acting substances so the withholding period can be adjusted if the dry period is shorter than expected.
- Withholding periods after treatment of cows with veterinary drugs should be regarded in any case. The excretion period is dependent on antibiotic and vehicle used in a certain drug. Therefore the withholding time is valid for the specific drug. Different withholding periods may be appropriate for two drugs containing the same antibiotic.
- To prevent prolonged excretion of residues in milk cows should be milked at least twice per day. This is not only desirable with regard to withholding periods but especially in case of mastitis to remove the secretion from the diseased udder quarter. It should be considered that cows with mastitis may visit the AM system less frequently than usual due to pain during milking or general symptoms of disease.
- Highest concentrations of antibiotics in milk are expected during the first milkings after treatment. The risk of bulk tank contamination can be reduced if during this time treated cows are milked before the main cleaning cycle under supervision of the farmer. This would also facilitate the control of treatment success for the farmer.
- Recommendations of the drug manufacturer regarding dosage, treatment intervals, route of administration and storage of veterinary drugs should be followed closely because any deviation may lead to extended withholding periods.
- Milk from untreated quarters must not be delivered because transfer of antibiotics from treated to untreated quarters may occur.

- Before milk of treated cows is delivered to the bulk tank after the end of the withholding period the success of treatment should be checked, e.g. by California Mastitis Test to prevent delivery of milk with high SCC to the bulk tank.
- If cow-side tests are applied to make sure that milk of individual cows is free of inhibitors after the end of the withholding period, farmers should be aware of the fact that screening tests do not detect all antibiotics with sufficient sensitivity. Test systems detecting the applied drug at MRL-level should be used.
- The efficiency of cleaning of milking equipment should be checked in regular intervals if automatic cleaning procedures are used after milking of treated cows. Recommendations of the AM manufacturer with regard to maintenance of the system should be closely followed to avoid cleaning failures.
- The application of appropriate mastitis control programmes will help to reduce the incidence of mastitis and thus the need for antibiotic treatment in dairy cows.

#### **Recommendation for the veterinarian**

- Veterinarians should be aware of the specific aspects of AM when treating dairy cows. For necessary treatments a suitable procedure should be agreed upon with the farmer to prevent accidental milking of cows after treatment (see above).
- For antibiotic treatments guidelines for prudent use of antibiotics should be followed.
- The veterinarian should supply appropriate information on treatment of individual animals and remind the farmer of the appropriate withholding periods.

#### **Recommendation for the AM manufacturer**

- The manufacturer should be aware of the high demands on automated cleaning procedures to prevent carry over of residues. Appropriate function of the cleaning procedures should be ensured. This includes also the prevention of carry over via the teat cleaning device after contamination with milk from a treated cow due to milk leaking.
- For carry over experiments in development of suitable cleaning procedures concentrations of antibiotics in milk should be used according to expectations from worst case studies. For detection of antibiotic residues in milk sampled after cleaning procedures test systems should be used which detect the respective antibiotic at MRL level. Preferably antibiotic residues should be quantified. This is also recommended for other investigators.
- During regular visits for maintenance of the system special attention should be directed to checking of the cleaning procedure.
- To facilitate record keeping for the farmer it is desirable to provide means for long term identification of treated cows either in the AM programme itself or by exchange of information with an existing management programme.

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## Abbreviations

AM	Automatic Milking
<i>B. stearothermophilus</i>	<i>Bacillus stearothermophilus</i> (today <i>Geobacillus stearothermophilus</i> )
CEF	cefquinome
CNS	coagulase-negative staphylococci
<i>E. coli</i>	<i>Escherichia coli</i>
h	hour(s)
I.U.	International Unit
LF	left front
LH	left hind
LSQ <sub>M</sub>	least square mean
MRL	Maximum Residue Limit
n.a.	not available
n.d.	not determined
RF	right front
RH	right hind
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>Sc. uberis</i>	<i>Streptococcus uberis</i>
se	standard error
SCC	somatic cell count
XQ	arithmetic mean