Influence of milking frequencies in automatic milking systems on excretion characteristics of different antibiotics in milk

by K. Knappstein, G. Suhren and H.-G. Walte

Institute for Hygiene and Food Safety, Federal Research Centre for Nutrition and Food – Location Kiel, Hermann-Weigmann-Str. 1, D-24103 Kiel

1. Introduction

Prevention of residues of antibiotic drugs in milk is necessary to ensure health protection of consumers and to avoid failures during milk processing. Bulk tank milk is routinely screened for inhibitors including antibiotic residues. The detection of inhibitors within quality payment schemes has severe economic consequences for the farmer resulting in decreasing numbers of positive findings (36). Treatment of mastitis with antibiotics is one of the most important causes of positive results of inhibitor tests on bulk tank milk (15). In conventional milking inhibitors in milk are mainly due to failures in management, e.g. not attending the withholding time, accidental milking of treated cows, wrong milking order. In addition, technical failures may lead to insufficient cleaning of milking equipment (32). Rasmussen *et al*. (28) reported a higher risk for positive inhibitor tests to be associated with Automatic Milking (AM) systems, especially in the first months after introduction of these systems on dairy farms. Like in conventional milking management failures were determined as main reasons for residues of antibiotics in milk. In a small percentage of events no reason could be detected. In these cases prolonged excretion of residues in milk of treated cows may be suspected among other reasons.

Excretion of residues after intramammary treatment of cows is most likely affected by different milking intervals. Although the objective of AM is to milk cows more frequently than twice daily also irregular and prolonged milking intervals are observed in individual cows. Limited information is available on the effects of milking frequency on the excretion of antibiotic residues in milk. Available studies mainly dealt with the influence of frequent stripping after treatment of cows with clinical mastitis on concentrations of antibiotics in milk (18). Therefore this study focuses on excretion characteristics of different antibiotics in milk after intramammary treatment of lactating cows in dependence on milking frequency.

In the European Union regular milking intervals twice a day are the basis for determining withdrawal periods for milk after treatment of healthy cows with veterinary drugs (12). The determination is based on the time necessary to fall short of the maximum residue limits (MRLs) which are fixed according to Regulation 2377/90 EEC (6). Residues are determined in composite milk of individual cows (12). Because excretion patterns in cows with clinical mastitis are difficult to compare regarding severity of disease or mastitis pathogens involved, this study was performed with healthy cows.

Four different commercially available udder injectors containing six different antibiotics were used for the experiments. For each drug three groups of five cows were milked with different milking frequencies under experimental conditions. Samples were analysed by appropriate screening and liquid chromatography (LC)-methods for identification and

quantification of residues. The time necessary for antibiotic residues in milk to fall short of the MRLs was determined for the three groups. The excretion time in milk was compared to the indicated withholding period for the drugs applied.

2. Literature review

2.1 Influence factors on excretion of residues in milk

Apart from the milking frequency different factors with influence on the excretion of antibiotic residues in milk have to be regarded.

2.1.1 Physico-chemical properties of antibiotics used in mastitis therapy

For effective treatment of mastitis it is important to reach high concentrations of antibiotics in the udder tissue. Because of the blood udder barrier and the pH difference between milk (pH 6.5-6.8) and blood serum (pH 7.4), the distribution of antibiotics is dependent on physico-chemical properties. Biological membranes like the blood udder barrier are only permeable for the non-ionized, lipid soluble part of drugs (48). Weak acids like benzylpenicillin are mainly ionized if the pH is higher than the pK_a-value. In healthy cows the concentration in blood is higher than in milk because with increasing pH a larger part of the substance is ionized. In contrast, weak bases like macrolides are mainly non-ionized at blood pH and can permeate the blood udder barrier. In milk the major part is ionized which leads to an enrichment of these substances in milk (7, 10, 48). With increasing pH during clinical mastitis weak acids reach higher concentrations in milk than in healthy udders due to restricted diffusion of non-ionized particles (48). A high degree of binding to udder tissue and/or milk proteins reduces the availability of antibiotics and affects the excretion after treatment (7).

Properties of the most important antibiotics applied for mastitis treatment are summarized in table 1.

Only macrolides, lincosamides and some tetracyclines reach higher concentrations in milk of healthy cows than in blood serum.

However, the selection of antibiotics for therapy is also dependent on the antibacterial properties of the drug. Penicillins and macrolides are only effective against gram-positive bacteria except the broad spectrum penicillins like ampicillin or amoxicillin which also have an effect against gram-negative bacteria. Cephalosporines have an extended spectrum compared to penicillins, but this is dependent on the type of cephalosporine. Aminoglycosides and tetracyclines are broad spectrum antibiotics and effective against gram-positive and gram-negative bacteria whereas polypeptides are mainly effective against gram-negatives.

Tab. 1: Chemical properties and milk to serum concentration ratio of antibiotics parenterally applied to cattle (adapted from ZIV (48))

Antibiotic	Chemical nature	pK_a	Degree of lipid solubility	Milk to serum concentration ratio	
				normal milk	mastitic milk
β-lactam antibiotics					
Penicillins					
Benzylpenicillin	acid	2.7	moderate	0.12 - 0.20	0.25 - 0.32
Cloxacillin	acid	2.7	high	0.20 - 0.24	0.30 - 0.48
Ampicillin	acid	2.7, 7.2	high	0.22 - 0.30	0.28
Cephalosporines					
Cephaloridine[1]	acid	3.4	moderate	0.24 - 0.28	n.a.
Cephaloglycin[1]	acid	4.9	moderate	0.32 - 0.34	n.a.
Cefquinome	acid	2.5, 2.9	low	n.a.	n.a.
Aminoglycosides					
Dihydrostreptomycin, Gentamicin, Neomycin, Kanamycin	base	9.0	low	0.20 - 0.80	0.35 - 0.70
Macrolides					
Erythromycin	base	8.8	high	3.00 - 4.00	1.80 - 2.60
Tylosin	base	7.1	high	3.60 - 5.40	1.00 - 1.40
Spiramycin	base	8.0	high	4.20 - 4.80	2.60 - 3.30
Lincosamides					
Lincomycin, Clindamycin[1]	base	7.6	high	2.70 - 6.40	2.00 - 3.00
Polypeptides					
Polymyxin B[1], Colistin	base	10.0	low	0.20 - 0.40	0.38 - 0.70
Tetracyclines					
Tetracycline, Chlor-tetracycline, Dimethyl-chlortetracycline[1]	amphoteric	8.3, 10.2	low	0.92 - 1.28	n.a.

[1] application not allowed for food producing animals in the EU; n.a. = not available

2.1.2 Drug formula

The drug formula has significant influence on the distribution of antibiotics within the udder and the excretion time in milk (1, 9, 22, 25). Sustained release-formulations where the molecules are incorporated into a particular matrix can achieve a certain concentration over a prolonged time period. According to Edwards (9) penicillin injected in oily suspensions was retained for longer periods than in oil-in-water emulsions. The shortest retention time was observed for aqueous solutions (9). The injected volume did not influence the concentrations of penicillin in the udder, whereas Moretain and Bisseau (25) found the elimination to be dependent on the volume of ointment infused.

2.1.3 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 (9) found irregular distribution of penicillin in udder quarters during later stages of lactation. This was explained by an increase in connective 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.

Schluep *et al.* (34) observed that the elimination of cefacetrile was slightly retarded in experimentally infected quarters when a slow release formulation was applied for intramammary treatment. 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) (11). 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 (30). For cefotaxime concentrations in milk of healthy quarters were only higher at 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 (50). 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 µg/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 pH>7.2 than in milk with pH<7.2 after intramammary or intravenous application (27). The excretion period in milk was shorter with more severe abnormalities of milk. These findings were consistent with those from Schällibaum *et al*. (33) who reported lower concentrations in mastitic milk than in milk of healthy cows after intramammary application of spiramycin.

Malvisi *et al*. (24) 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 after intramammary infusion.

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 (49). 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.

From the literature review it becomes obvious that the reports on antibiotic concentrations in milk and length of depletion time in dependence on udder health status vary.

2.1.4 Other influence factors on excretion of residues in milk

The findings reported on influence of milk yield on milk depletion time of different antibiotics were not consistent (4, 29, 35, 46). Probably only extreme low milk yields lead to a longer excretion period in milk. For penicillin excretion no relation was found with fat content of milk and body weight of the treated cow (4). Seymour *et al*. (35) also found no significant influence of neither milk yield and body weight nor of route of administration, case number, number of days treated, and lactation number. Koppinen *et al*. (22) determined a significant influence of total dosage applied, the product used and the number of days in lactation.

2.2 Maximum Residue Limit (MRL) and withdrawal period for milk

In the EU MRLs for veterinary drugs in animal tissues and milk are based on the Acceptable Daily Intake (ADI) for the consumer calculated from the NOEL (No Observable Effect Level) and a safety factor (47). Safety considerations include toxicity, teratogenicity, mutagenicity, carcinogenicity and sensitizing potential for allergic reactions (immunotoxicity). In addition, the NOELs with respect to the human gut flora and – in the case of milk – starter cultures used in the dairy industry are taken into account. MRLs in milk are calculated on the basis of a consumption of 1.5 liters of milk per person and day. In table 2 the MRLs in milk according to Regulation 2377/90 EEC (6) are listed.

According to Directive 81/851 EEC (5) the withdrawal period is defined as the interval between the last administration of a veterinary medicinal product to animals under normal conditions of use and the production of foodstuff from such animals to ensure that such foodstuffs do not contain residues in quantities in excess of maximum residue limits laid down (5). Since the predominant milking scheme is twice per day, experiments for the determination of withdrawal periods for milk should be carried out with animals milked accordingly. In the EU, guidelines exist for the determination of withdrawal periods for milk. For all milk producing species withdrawal periods are established for individual animals and not for bulk tank milk because milk from individual or few animals is used for consumption as well as for small-scale production of dairy products on farm level. From a statistical perspective, a sample size of 19 cows is the very minimum requirement for experiments to establish withdrawal periods. For products intended for intramammary treatment all quarters should be treated in order to represent a worst case situation (12).

2.3 Antibiotics used for treatment trials

Limited information is available on the kind and amount of antibiotics used in dairy cows in European countries. According to data from Denmark penicillins and cephalosporines alone or in combination with others accounted for about 95 % of intramammary treatments in 1998 and 1999 with a total of 500 kg active substance in 1999 (26). These data are consistent with estimations from Germany indicating that for lactational therapy mainly β-lactam-antibiotics (penicillins and cephalosporines) are applied. Aminoglycosides (neomycin, gentamicin, dihydrostreptomycin or kanamycin), lincomycin and colistin are of minor importance (43).

Tab. 2: MRLs (µg/kg) of residues of antimicrobial drugs in milk according to Regulation 2377/90 EEC (January 2006 (6))

Substance(-group)	MRL	Substance(-group)	MRL
β-Lactams		**Aminoglycosides**	
Benzylpenicillin	4	Gentamicin	100 [10]
Penethamat	4 [1]	Kanamycin	150
Ampicillin	4	Neomycin incl.	
Amoxicillin	4	Framycetin	1500 [11]
Nafcillin	30 [2]	Spectinomycin	200
Cloxacillin	30	Dihydro-/Streptomycin	200
Dicloxacillin	30 [3]		
Oxacillin	30	**Sulfonamides**	100 [12]
Cefacetrile	125 [2]		
Cefalexin	100	**Quinolones**	
Cefalonium	20	Danofloxacin	30
Cefoperazon	50	Enrofloxacin	100 [13]
Ceftiofur	100 [4]	Flumequin	50
Cefquinom	20	Marbofloxacin	75
Cephapirin	60 [5]		
Cephazolin	50	**Various**	
		Bacitracin	100 [14]
Tetracyclines		Baquiloprim	30
Chlortetracycline	100 [6]	Clavulanic acid	200
Oxytetracycline	100 [6]	Colistin	50
Tetracycline	100 [6]	Lincomycin	150
		Novobiocin	50
Macrolides		Pirlimycin	100
Erythromycin	40 [7]	Rifaximin	60
Spiramycin	200 [8]	Thiamphenicol	50
Tilmicosin	50	Trimethoprim	50
Tylosin	50 [9]		

[1] marker benzylpenicillin,
[2] only for intramammary treatment,
[3] marker cloxacillin,
[4] sum of all residues retaining β-lactam structure expressed as desfuroylceftiofur,
[5] sum of cefapirin and desacetylcefapirin,
[6] sum of parent drug and 4-epimer,
[7] marker erythromycin A,
[8] sum of spira- and neospiramycin,
[9] tylosin A,
[10] sum of gentamicin C1, C1a, C2 and C2a,
[11] neomycin B,
[12] sum of all substances of this group,
[13] sum of enro- und ciprofloxacin,
[14] sum of bacitracin A, B and C

Due to European regulations a large number of veterinary drugs has lost approval at the end of June 2003, limiting the availability of certain antibiotics for dairy cow treatment.

Based on the available information on usage of antibiotics in mastitis therapy and the different pharmacokinetics of antibiotics four different intramammary devices containing six antibiotic drugs alone or in combinations were selected for the treatment trials. In the following the most important aspects are summarized for the antibiotics included in this study.

2.3.1 β-Lactam antibiotics

Penicillins (PEN) are the most important group of antibiotics. The toxicity of these substances is low. The main excretion of penicillin is via urine, partly also via bile. Metabolism is of little importance in the elimination of penicillins. Penicillins can produce allergic reactions in humans which is the most important side-effect in the use of penicillins. For inducing an allergic reaction a much higher oral dose is needed compared to parenteral administration (13). Starter cultures used in milk processing are very sensitive for penicillins. For some penicillins concentrations below the MRL have significant influence on acid production by starter cultures (23, 38, 44).

Procain-penicillin is a depot penicillin with sustained release of benzylpenicillin.

Ampicillin (AMP) is acid-stable with low protein binding. There are no indications that starter cultures are significantly influenced by AMP at concentrations ≤ MRL.

Nafcillin (NAF) is a penicillinase-resistant penicillin. After intramammary application NAF is systemically resorbed, but in lactating cows the main part is excreted via milk. The activity of a commercially available yoghurt culture was significantly influenced at concentrations ≤ MRL (38).

For cefquinome (CEF), a cephalosporine, low systemic resorption occurs after intramammary application. Excretion is mainly via milk. Metabolism is of low importance. The NOEL for *Streptococcus thermophilus* – a bacterium used in starter cultures – is 20 µg/kg corresponding to the MRL (14), but a significant influence on the activity of a commercially available yoghurt culture was observed at concentrations ≤ MRL (38).

2.3.2 Dihydrostreptomycin (DHS)

DHS is a basic substance with high polarity. At physiological pH DHS is in ionized form with low penetration of biological membranes. Protein-binding is low. Excretion is dependent on the drug formula. There are no hints for metabolism of DHS in food-producing animals. The activity of a commercially available yoghurt culture was significantly influenced at concentrations ≤ MRL (38).

2.3.3 Colistin (COL)

COL is a polar substance with hydrophobic properties and is highly irritating after intramuscular administration. Protein-binding is reduced with increased dose. COL is only active against gram-negative organisms, whereas gram-positive bacteria which are used as starter cultures in the dairy industry are not affected.

3. Materials and Methods

3.1 Set up of experiments

3.1.1 Parameters for characterization of cows

German Holstein, black and white, from the experimental station Schaedtbek of the Federal Research Centre for Nutrition and Food were used for the excretion trials. The experiments were performed with permission of the competent authority for animal welfare. The following parameters were used for selection and characterization of cows used in the treatment trials:

- Number of lactation
- Days in milk
- Average milk yield per day during experimental period in kg
- Udder health status
- Body weight in kg (only determined in Nafpenzal® MC and Omnygram® trials)

Cows with comparable milk yield per day were used for treatment trials with different milking frequencies. Somatic cell count (SCC) in cow composite milk was below 100.000/ml in 3 weekly investigations before the start of experiments and cows had not been treated with antimicrobials within a time period of 4 weeks before start of experiments.

3.1.2 Milking frequencies

For the treatment trials groups consisting of 5 (in two exceptions 4 and 6 respectively) cows were milked in a tandem milking parlour with three different milking frequencies per drug:

- 2 times per day at 4.30 a.m. and 2.30 p.m., milking intervals of 10 and 14 hours (reference group)
- 3 times per day at 4.00 a.m., 12.00 and 8.00 p.m., milking intervals of 8 hours, adjustment of cows to higher milking frequency 7 days before start of treatments
- 1.5 times per day at 4.00 a.m. and 8.00 p.m. every second day and at 12.00 every other day, milking intervals of 16 hours

Within each trial three milkings before the first treatment were used as anamnesis.

3.1.3 Sampling

Sampling was performed during the experimental period consisting of anamnesis, treatment period, withholding period for milk plus two additional days. If antibiotic residues in milk were detected more than two days after the end of the withholding period the experimental period was prolonged until samples of the respective cows reacted negative in screening tests during at least three successive milking times.

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

- Quarter milk samples (at the beginning of milking)
  - 10 ml, preservation with potassium dichromate (0.2 %) for determination of SCC
  - once during anamnesis 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 until analysis at 6 °C for 60 hours at maximum. For further investigations cow composite milk samples were stored at -20 °C for 3 weeks at maximum. Samples for later re-examinations were preserved by lyophilization and stored at 6 °C.

Tab. 3: Antibiotic drugs used for treatment trials

Drug	Cobactan® LC	Procain-Penicillin G 3 Mio. Euter-injektor	Nafpenzal® MC	Omnygram®
Manufacturer	Hoechst Roussel Vet[1], Unterschleissheim, DE	WDT, Garbsen, DE	Intervet Int., Boxmeer, NL	Virbac S.A., Carros, France
Composition	88.8 mg Cefquinome (CEF)-sulfate = 75 mg CEF	3 g Procainbenzylpenicillin = 1898 mg Penicillin G (PEN)	300 000 I.U. Penicillin (PEN)-sodium = 180 mg PEN; 122 mg Nafcillin (NAF)-sodium = 100 mg NAF; 134 mg Dihydrostreptomycin (DHS)-sulfate = 100 mg DHS	1000 mg Ampicillin (AMP)-trihydrate = 866 mg AMP; 2 500 000 I.U. Colistin (COL)-sulfate = 82.5 mg COL[2]
Total volume per injector	8 g	20 g	3 g	10 ml
Lot-No., Expiry date	01H033, 02/2003; 01H013, 09/2002 for repeated experiment with milking 2x per day	093090, 03/2002	not available	083B, 03/2004
Withholding time for milk	5 days	5 days	NL: 5 days UK: 3 days	6 days
MRL for milk	20 µg/kg	4 µg/kg	PEN: 4 µg/kg NAF: 30 µg/kg DHS: 200 µg/kg	AMP: 4 µg/kg COL: 50 µg/kg
Dosage	3 intramammary treatments, 4 quarters per cow (worst case), one injector per quarter per treatment was applied			
Recommended treatment scheme	3 treatments during successive milking times	3 treatments with intervals of 24 hours	3 treatments with intervals of 24 hours	Treatment every 12 hours with at least one treatment after improvement of clinical symptoms
Recommended storage	below 30 °C	below 20 °C	at 2°C - 25 °C	at 12°C - 15°C
Total amount of pure substance applied per cow (3x4 injectors)	900 mg CEF	22 776 mg PEN	2160 mg PEN 1200 mg NAF 1200 mg DHS	10392 mg AMP 990 mg COL

[1] today Intervet International; [2] 1 IU = 0.033 µg COL salt (45)

3.1.4 Drugs

For treatment trials commercially available udder injectors were used (table 3).

3.1.5 Treatment intervals

Treatment schemes as summarized in table 4 were used to apply the same quantity of antibiotic substance per time period. In the Omnygram® experiment with milking frequency of 2 times per day cows were treated at three successive milking intervals as recommended by the manufacturer. In the other two treatment trials cows were treated 3 times within 48 hours.

Tab. 4: Treatment schemes

	Milking frequency		
	3 times per day	**2 times per day**	**1.5 times per day**
Cobactan® LC and Omnygram® [1]			
Anamnesis	day 1, 4.00 h	day 1, 14.30 h	day 1, 4.30 h
	day 1, 12.00 h	day 2, 4.30 h	day 1, 14.30 h
	day 1, 20.00 h	day 2, 14.30 h	day 2, 4.00 h
Treatment:			
3 times per 24 hours	day 1, 20.00 h	day 2, 14.30 h	day 2, 4.00 h
	(day 1, 4.00 h)	day 3, 4.30 h	day 2, 12.00 h
	day 2, 12.00 h	day 3, 14.30 h	day 3, 4.00 h
	day 2, 20.00 h		(day 3, 20.00 h)
Procain-Penicillin G 3 Mio. Euterinjektor, Nafpenzal® MC and Omnygram® [2]			
Anamnesis	day 1, 4.00 h	day 1, 14.30 h	day 1, 14.30 h
	day 1, 12.00 h	day 2, 4.30 h	day 2, 4.30 h
	day 1, 20.00 h	day 2, 14.30 h	day 2, 20.00 h
Treatment:			
3 times per 48 hours	day 1, 20.00 h	day 2, 14.30 h	day 2, 20.00 h
	(day 2, 4.00 h)	(day 3, 4.30 h)	(day 3, 12.00 h)
	(day 2, 12.00 h)	day 3, 14.30 h	day 4, 4.00 h
	day 2, 20.00 h	(day 4, 4.30 h)	day 4, 20.00 h
	(day 3, 4.00 h)	day 4, 14.30 h	
	(day 3, 12.00 h)		
	day 3, 20.00 h		

Milking times in brackets: no treatment;
[1] only milking frequency 2 times per day,
[2] only milking frequencies 3 and 1.5 times per day

3.2. Analytical methods

3.2.1 Udder health

SCC was determined according to IDF Standard 148A:1995, Method C, Fluoro-opto-electronic method (19). The bacteriological investigation was performed according to guidelines of the German Veterinary Association (8).

3.2.2 Qualitative detection of antibiotic residues

For screening purposes and for determining the end of the sampling period appropriate screening methods were applied.

Microbiological inhibitor tests:

The following commercially available microbial inhibitor tests with *Bacillus (B.) stearothermophilus*[1] as test microorganisms were used:

- Delvotest SP (DSM Food Specialities, Dortmund, DE)
- BR-Test AS (DSM Food Specialities, Dortmund, DE)
- BR-Test AS special (DSM Food Specialities, Dortmund, DE)
- BR-test AS Brilliant (DSM Food Specialities, Dortmund, DE)
- BRT-Hemmstofftest (Chr. Hansen GmbH, Nienburg, DE)

Test performance was according to the instructions of the manufacturer. Visual reading as inhibitor and screening test was performed according to standard procedures (2, 3).

Preliminary confirmation test - Beta Star:

The commercially available Beta Star test (Chr. Hansen GmbH, Nienburg/DE) is a receptor assay for rapid detection of β-lactam antibiotic residues in milk.

The test is interpreted by visual comparison of a test band with a reference band. Results are coded in 4 levels; steps 2, 3 and 4 are interpreted as positive.

Determination of detection limits of microbial inhibitor and Beta Star tests

The description of IDF Standard 183/ISO 13969 (20) was followed as closely as adequate. The detection limits of spiked milk samples are summarized in table 5 (40).

Tab. 5a: Detection limits of screening tests (µg/kg) - Spiked samples

Test	CEF		PEN		NAF	
	Inhibitor test	Screening test	Inhibitor test	Screening test	Inhibitor test	Screening test
Delvo SP	150	100	2	1	15	7.5
BR-AS special	>250	>250	2	1	15	15
BRT	>250	150	3	2	15	15
BR-AS	n.d.	n.d.	4	3	n.d.	n.d.
BR-AS Brilliant	n.d.	n.d.	4	3	30	30
Beta-Star	12.5	-	2	-	30	n.d.

n.d. = not determined

[1] Now: *Geobacillus stearothermophilus* due to recent taxonomic studies

Tab. 5b: Detection limits of screening tests (µg/kg) - Spiked samples

Test	DHS		AMP		COL	
	Inhibitor test	Screening test	Inhibitor test	Screening test	Inhibitor test	Screening test
Delvo SP	5 000	2 500	6	4	50 000	50 000
BR-AS special	5 000	2 500	4	4	10 000	5 000
BRT	n.d.	n.d.	6	4	50 000	50 000
BR-AS	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BR-AS Brilliant	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Beta-Star	n.d.	n.d.	6	4	n.d.	n.d.

n.d. = not determined

Of the microbial inhibitor tests the most sensitive test for the detection of CEF is Delvotest SP. However, the detection limit is much higher than the MRL of 20 µg/kg. By the receptor test Beta-Star CEF can be detected at < MRL-level.

By all *B. stearothermophilus* tests and the Beta Star test the MRLs of PEN, NAF and AMP (4 µg/kg, 30 µg/kg and 4 µg/kg, respectively) can at least be detected when applied as screening test. Detection limits of *B. stearothermophilus* tests for DHS and COL are far above the MRLs of 200 µg/kg and 50 µg/kg, respectively.

3.2.2.5 Enzyme Linked Immunoassays (ELISA)

Microbial inhibitor tests showed insufficient sensitivity for detection of DHS and COL. Therefore ELISAs were used as screening tests for these two substances. The method describing criteria are summarized in table 6 (16, 17, 21, 37, 41).

Tab. 6: Criteria of ELISA methods applied

Criterion	DHS	COL
Recovery rate[1] (%)	128	146
Coefficient of variation[1]	16.3	11.7
LOD (µg/kg)[2]	13.7 [4]	10.9
LOQ (µg/kg)[3]	24.2 [4]	19.9

[1] Determined at concentrations of 100 µg/kg DHS and 50 µg/kg COL resp.;
[2] LOD = limit of detection;
[3] LOQ = limit of quantification;
[4] in STR-equivalents

3.2.3 Quantitative detection of antibiotic residues - HPLC methods

For the identification and quantification of DHS (37), CEF (39), AMP, NAF and PEN (42) and COL (41) published HPLC-methods were used. The experimental parameters are summarized in table 7. Evaluation is via linear regression between peak areas and concentrations of aqueous standard solutions considering the concentration factors and

the respective recovery rates. By analysis of negative raw milk samples the limit of detection (LOD = XQ + 3s; with XQ = arithmetic mean, s = standard deviation) and the limit of quantification (LOQ = XQ + 6s) are derived.

Tab. 7: Criteria of HPLC-methods applied

Criterion	CEF	PEN	NAF	DHS	AMP	COL
Recovery (in %)[1]	90	64	48[4]	90	71	106
Repeatability, s (µg/kg)[1]	0.9	0.4	1.1[4]	8.5	0.4	7.2
LOD (µg/kg)[2]	<2	<1	<1	29	<1	9
LOQ (µg/kg)[3]	<3	<1	1	54	1	14

[1] Level of MRL-concentrations [2] LOD = limit of detection [3] LOQ = limit of quantification [4] concentration 4 µg/kg

3.3 Determination of withdrawal time

Different approaches to determine the withdrawal periods were applied to compare the results of the experiments with different milking frequencies:

3.3.1 Pragmatic approach

– Pragmatic - average

For each experiment the mean concentration (µg/kg) of residues in milk was calculated for every sampling time from the concentrations in milk of individual cows as determined by HPLC-methods. The sampling time (in hours) after the last application of antibiotics was derived, when the average content fell below the respective MRL and did not exceed the MRL again. The mean was calculated from this time and the last 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 was calculated as mean from those values.

3.3.2 Time-to Safe-Concentration (TTSC) method

According to the TTSC-method described by a guidance document of the European Agency for the Evaluation of Medicinal Products for the determination of withdrawal periods for milk (12) a tolerance limit for the number of milkings per animal is calculated. 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 each experiment were calculated. According to the EMEA-procedure (12) 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 limit 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 individual results 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 (31), was used.

The first time when the content of residues in milk fell below the MRL was used as dependent variable (y). The following influence factors were included into the analysis: milking frequency per day (3, 2, 1.5), days in milk (=100 d, >100 d) and number of lactation (1, >1). Milk yield and SCC as continuous variables were used as covariate. The linear model had the following form:

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

With:

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

μ = overall mean

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

dim_j = effect of the j^{th} days in milk

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

b_1 = slope for milk yield

b_2 = slope for SCC

e_{ijkl} = random residual error

In addition, body weight (kg) was included as influencing factor for the Nafpenzal®MC and Omnygram® experiments.

4. Results

4.1 Cobactan® LC (Cefquinome)

4.1.1 Status of animals

The status of the cows included in the experiments with CEF-treatment is shown in table 8.

Cow No. 2158 was included into the trials with milking 2 times per day because of prolonged excretion in the experiment with milking 3 times per day. The experiment with milking 2 times per day had to be repeated because udder injectors for the second of 3 treatments were accidentally stored above 30 °C. One cow had to be excluded from the experiment with milking frequency 1.5 times per day after the anamnestic phase because of systemic illness.

Tab. 8: Cobactan® LC experiments – Status of animals

Cow	No. of lactation	Days in milk	Milk yield per day (kg)[1]	SCC in composite milk (1000/ml)[1]	Mastitis pathogens
Milking frequency 3 times per day					
1792	2	149	30.3	24	RH,LH: CNS
1835	1	114	27.3	50	n.d.
1839	1	137	37.8	44	RH: CNS
1852	1	113	35.4	48	RH: CNS
2158	6	268	25.2	136	n.d.
Milking frequency 2 times per day (mishandled injectors)					
1743	4	116	36.4	16	n.d.
1796	2	156	23.6	25	n.d.
1818	2	168	28.6	19	n.d.
1829	1	76	31.8	62	RH,LH: CNS
1833	1	186	24.6	42	RH,LH: CNS
2158	6	299	22.7	113	n.d.
Milking frequency 2 times per day (repetition of experiment with correctly handled injectors)					
1774	4	101	34.0	138	RH: CNS
1818	3	104	32.4	18	n.d.
1827	2	95	30.6	31	n.d.
1835	2	93	25.0	61	RF,LF,LH: CNS
1859	1	82	32.8	15	n.d.
Milking frequency 1.5 times per day					
1761	3	147	28.2	28	n.d.
1786	3	122	28.8	87	n.d.
1804	2	153	33.2	37	RF: CNS
1815	2	81	33.4	35	RH: CNS

[1] average during experimental period; n.d.= not detected; CNS = coagulase-negative staphylococci; quarters: RF = right front; LF = left front; RH = right hind; LH = left hind

4.1.2 Somatic cell count (SCC)

In figure 1 SCC in cow composite milk samples is summarized as average per group.

4.1.3 Excretion

In table 9 the excreted quantity of CEF via milk is summarized per cow and group with respect to the milking interval.

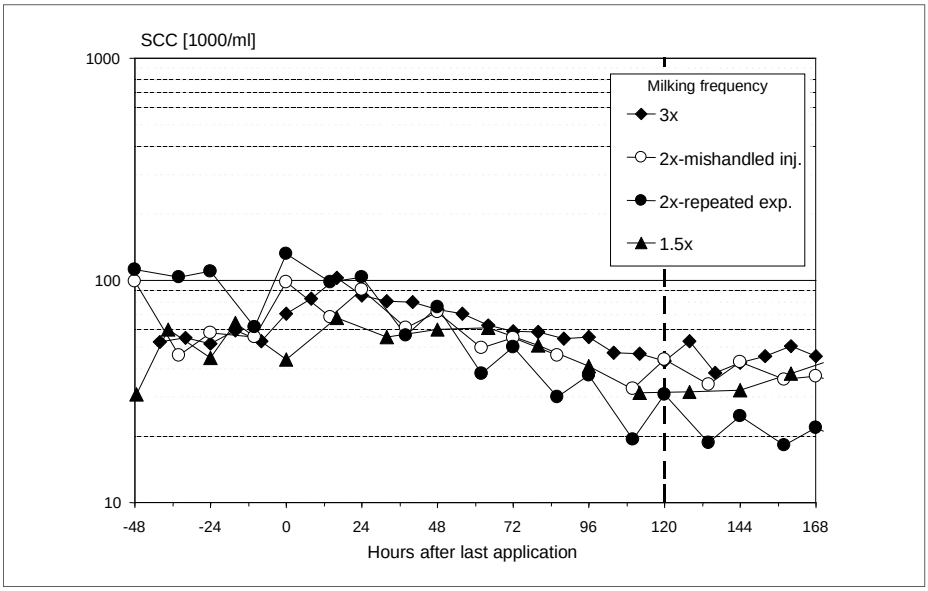

Fig. 1: SCC in milk in dependence on milking frequency - Cobactan® LC experiments – Average of 5 cows per group; — — — Withholding time

4.1.4 Withdrawal time

The excretion curves within each experiment are demonstrated for individual cows and as averages in figures 2 and 3. The quadratic and exponential regressions are presented in figure 4; in this figure the CEF-concentrations are transformed into *ln* values. The withdrawal times as determined by different approaches are summarized in table 10.

Tab. 9: Cefquinome excretion via milk in dependence of milking interval

Cow no.	Milk yield (kg)	Excreted CEF in mg	Excreted CEF in % of applied[1]
Milking frequency: 3 times per day			
1839	37.8	441	49.0
1852	35.4	592	65.8
1792	30.3	485	53.9
1835	27.3	305	33.9
2158	25.2	597	66.3
Average	**31.2**	**484**	**53.7**
Milking frequency: 2 times per day (mishandled injectors)			
1743	36.4	426	47.3
1829	31.8	337	37.5
1818	28.6	521	57.9
1833	24.6	341	37.9
1796	23.6	250	27.8
2158	22.7	597	66.3
Average	**28.0**	**412**	**45.8**
Milking frequency: 2 times per day (repeated experiment)			
1774	34.0	397	44.1
1859	32.8	465	51.7
1818	32.4	498	55.3
1827	30.6	406	45.1
1835	25.0	173	19.2
Average	**31.0**	**388**	**43.1**
Milking frequency: 1.5 times per day			
1815	33.4	468	52.0
1804	33.2	463	51.4
1786	28.8	367	40.8
1761	28.2	555	61.7
Average	**30.9**	**463**	**51.5**

[1] 100 % = 900 mg

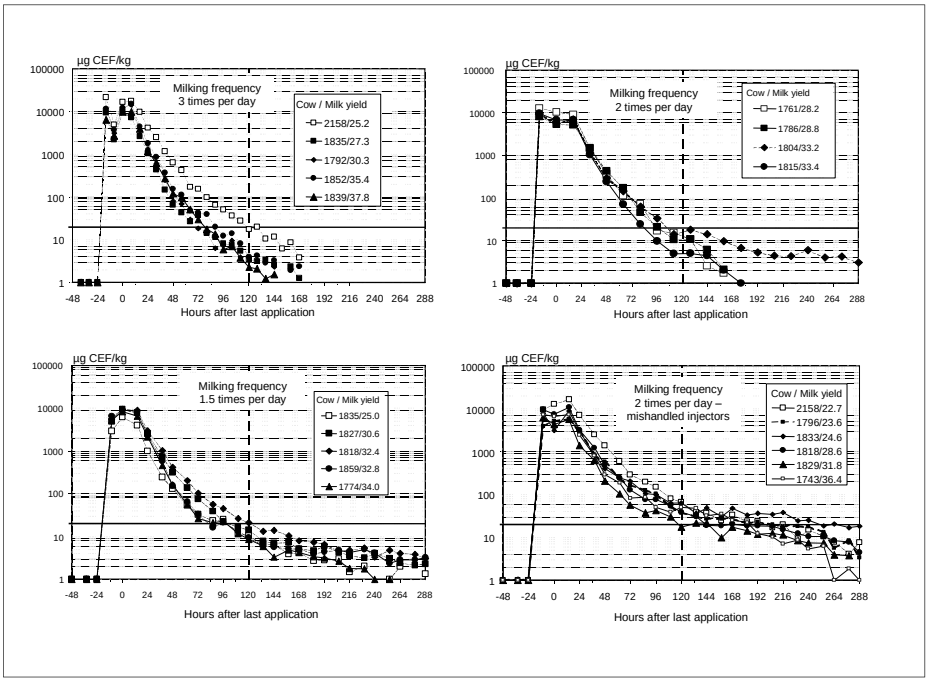

Fig. 2: Excretion of cefquinome (Cobactan® LC) in milk of individual cows; ———— MRL, — — — Withholding time

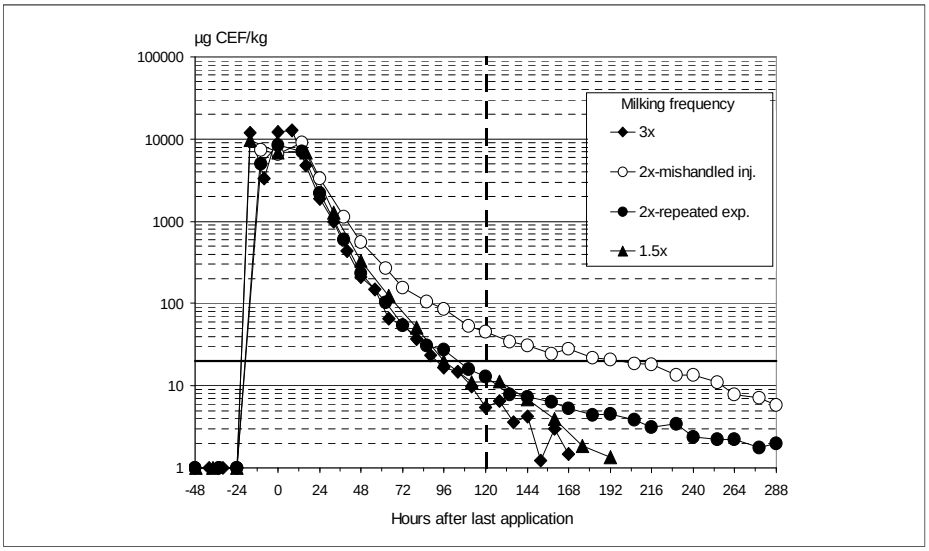

Fig. 3: Excretion of cefquinome (Cobactan® LC) in milk in dependence on milking frequency – Average of 5 cows per group; ———— MRL, — — — Withholding time

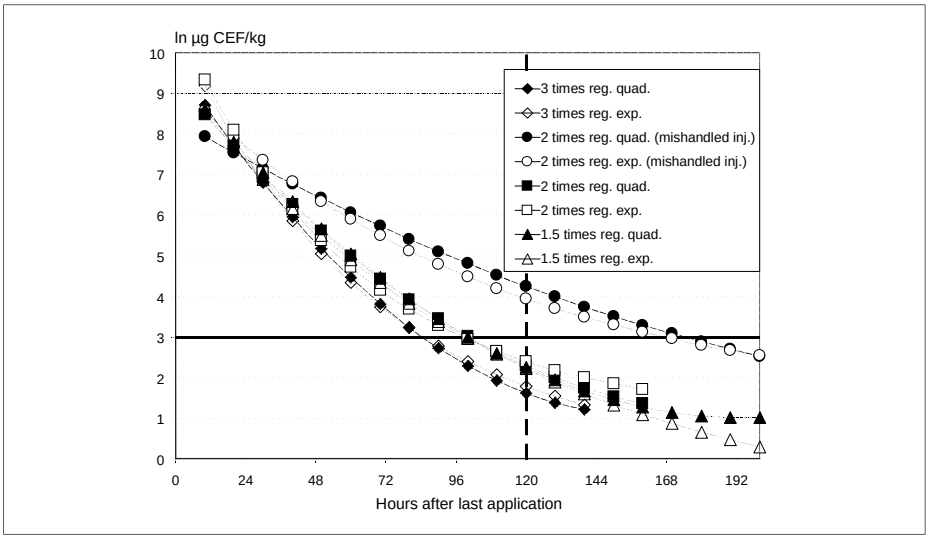

Fig. 4: Excretion of cefquinome (Cobactan® LC) in milk in dependence on milking frequency – Quadratic and exponential regressions; ——— MRL, — — — Withholding time

Tab. 10: Withdrawal time of CEF (in hours) in dependence on milking frequency – summarized results from pragmatic approach, TTSC and regression models; indicated withdrawal time: 120 h

Milking frequency	**3 times per day**		**1.5 times per day**	
Approach	Mean	95/95 tol. limit[1]/ 95% conf. int.[2]	Mean	95/95 tol. limit[1]/ 95% conf. int.[2]
Pragmatic - individual	88		96	
Pragmatic -average	92		104	
TTSC[1]	87	197	104	164
Quadratic regression[2]	84	121	100	137
Exponent. regression[2]	84	127	103	142
Milking frequency	**2 times per day**		**2 times per day** (mishandled injectors)	
Approach	Mean	95/95 tol. limit[1]/ 95% conf. int[2]	Mean	95/95 tol. limit[1]/ 95% conf. int.[2]
Pragmatic - individual	108		199	
Pragmatic - average	103		199	
TTSC[1]	109	211	187	547
Quadratic regression[2]	102	132	174	298
Exponent. regression[2]	97	129	165	305

[1] 95/95 tolerance limit, [2] 95 % confidence interval

4.1.5 Analysis of variance

The data from the experiment with mishandled injectors were excluded from the analysis of variance.

The model accounted for 63 % of the deviance of excretion time, but none of the predictors was significant. The differences between milking frequencies were therefore not significant (see table 11). The experiment with 3 milkings per day had the shortest withdrawal time, but there was no constantly decreasing withdrawal time with increasing milking frequency. Milking twice a day showed a longer withdrawal time than with 1.5 milkings per day.

Tab. 11: Withdrawal time (in hours) for cefquinome (Least Square Means (LSQ_M) and standard error(se))

Milking frequency	$LSQ_M \pm se$[1]	Cow no. 2158 excluded $LSQ_M \pm se$[1]
3 times	80.3 ± 20.0 [a]	71.8 ± 6.5 [a]
2 times	108.8 ± 11.1 [a]	111.6 ± 4.8 [b]
1.5 times	95.9 ± 15.5 [a]	99.9 ± 5.6 [b]

[1] Different superscripts show significant differences ($p<0.05$)

The analysis was repeated without data of cow no. 2158, which showed a markedly deviating excretion time from the other cows in the group milked 3 times per day (see figure 2). Using only the milking frequency and the days in milk, the generalized linear model accounted for 73 % of the variance. There were significant differences ($p<0.01$) between the milking frequency 3 times per day and the other tested frequencies, but the difference between 2 and 1.5 milking per day was not significant (table 11). The difference between the classes for the days in milk was slightly significant ($p<0.1$). The group in early lactation (<100 days in milk) reached the concentration below the MRL faster than the cows in the other class ($LSQ_M \pm$ se: 86.2 ± 6.2 to 102.7 ± 3.7).

According to the analysis of variance SCC had no significant influence on the excretion time. Therefore no further analysis was performed on the mastitis pathogens involved.

4.2 Procain-Penicillin G 3 Mio. Euterinjektor

4.2.1 Status of animals

The status of the cows included in the penicillin treatment trials is summarized in table 12.

4.2.2 SCC

The SCC in cow composite milk is shown as average per group in figure 5.

4.2.3 Excretion

In table 13 the excreted quantity of PEN via milk is summarized per cow and group with respect to the milking interval.

Tab. 12: Procain-Penicillin G 3 Mio. Euterinjektor experiments – Status of animals

Cow	No. of lactation	Days in milk	Milk yield per day (kg)[1]	SCC in composite milk (1000/ml)[1]	Mastitis pathogens
Milking frequency 3 times per day					
1731	4	162	33.9	43	n.d.
1810	2	176	23.5	58	LH: CNS
1827	1	133	29.1	36	n.d.
1831	1	138	38.6	26	n.d.
1834	1	96	32.9	53	RH: CNS
Milking frequency 2 times per day					
1796	3	41	31.4	21	n.d.
1833	2	110	33.6	26	n.d.
1869	1	46	29.0	34	n.d.
1870	1	56	32.7	25	n.d.
1880	1	54	24.4	26	n.d.
Milking frequency 1.5 times per day					
1697	5	111	32.4	17	n.d.
1716	5	82	33.1	391	n.d.
1763	3	94	32.5	34	n.d.
1824	1	190	26.9	25	n.d.
1848	1	215	22.6	71	n.d.

[1] average during experimental period, n.d. = not detected, CNS = coagulase-negative staphylococci; quarters: RF = right front, LF = left front, RH = right hind, LH = left hind

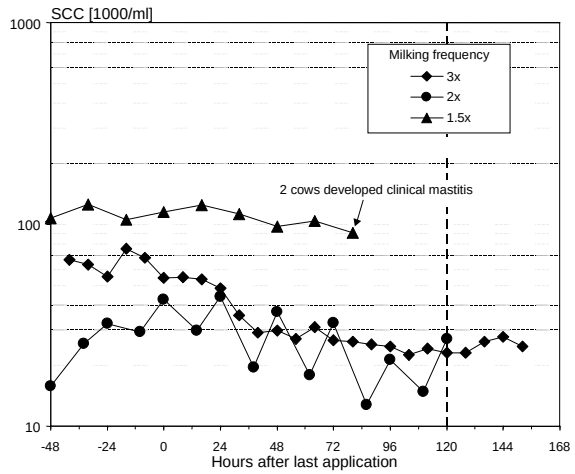

Fig. 5: SCC in milk in dependence on milking frequency – Procain Penicillin G 3 Mio. Euterinjektor experiments – Average of 5 cows per group; — — — Withholding time

Tab. 13: Penicillin excretion via milk in dependence on milking interval

Cow no.	Milk yield (kg)	Excreted PEN mg	Excreted PEN in % of applied[1]
Milking frequency: 3 times per day			
1831	38.6	9 244	40.6
1731	33.9	13 060	57.3
1834	32.9	8 760	38.5
1827	29.1	11 055	48.5
1810	23.5	8 293	36.4
Average	**31.6**	**10 078**	**44.2**
Milking frequency: 2 times per day			
1833	33.6	7 439	32.7
1870	32.7	8 206	36.0
1796	31.4	5 474	24.0
1869	29.0	6 548	28.7
1880	24.4	10 190	44.7
Average	**30.2**	**7 572**	**33.2**
Milking frequency: 1.5 times per day			
1763	32.5	9 695	42.6
1716	33.1	9 793	43.0
1697	32.4	7 654	33.6
1824	26.9	7 856	34.5
1848	22.6	7 042	30.9
Average	**29.5**	**8 408**	**36.9**

[1] 100 % = 22 776 mg

4.2.4 Withdrawal time

In figure 6 the excretion curves of the individual cows within each experiment and in figure 7 the average curves are demonstrated. The quadratic and exponential regressions for each experiment with *ln*-transformed concentrations are summarized in figure 8. The results of the determination of withdrawal times by the different approaches are compared in table 14.

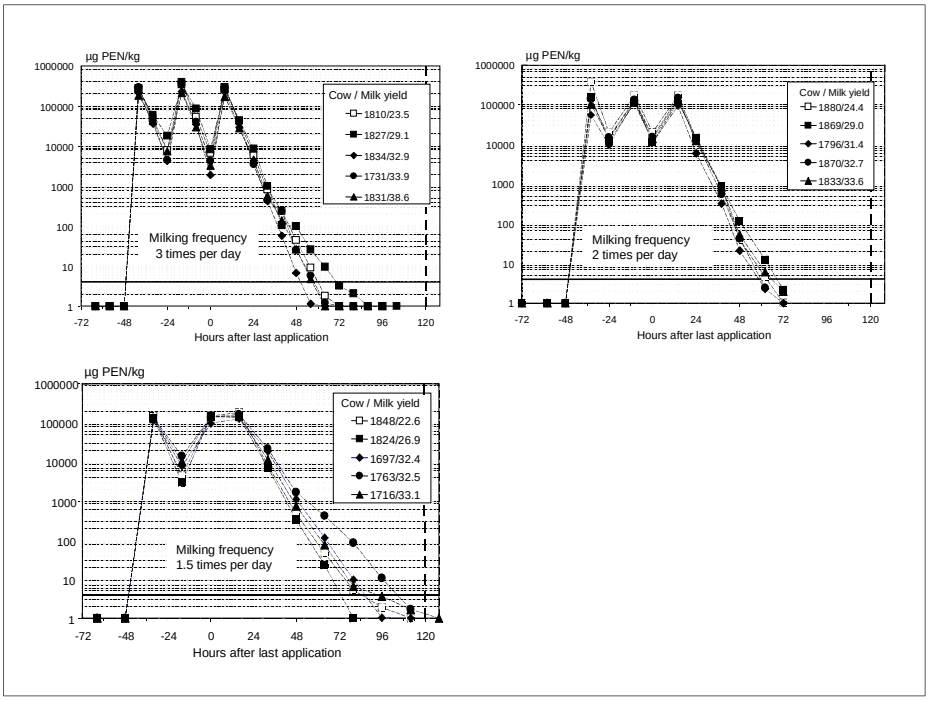

Fig. 6: Excretion of penicillin (Procain-Penicillin G 3 Mio. Euterinjektor) in milk of individual cows; ———— MRL, — — — Withholding time

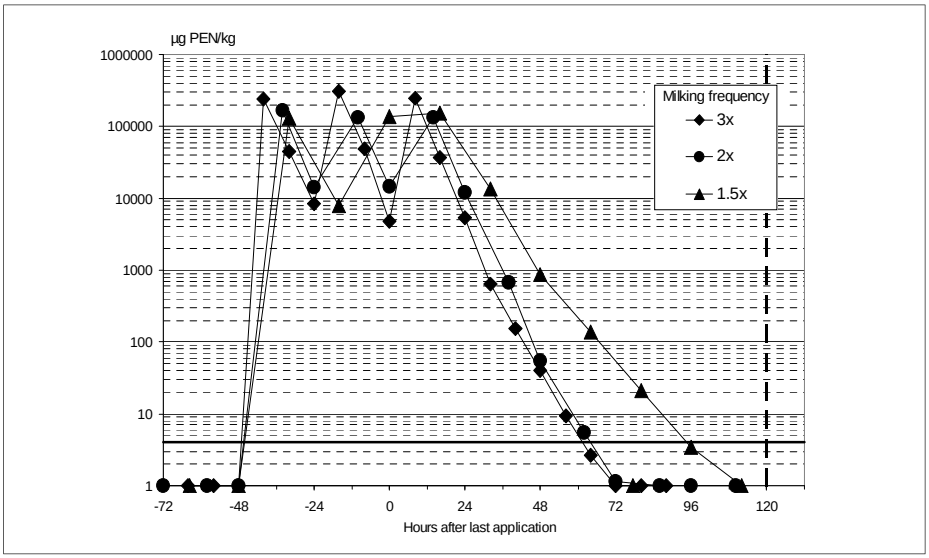

Fig. 7: Excretion of penicillin (Procain-Penicillin G 3 Mio. Euterinjektor) in milk in dependence on milking frequency – Average of 5 cows per group; ———— MRL, — — — Withholding time

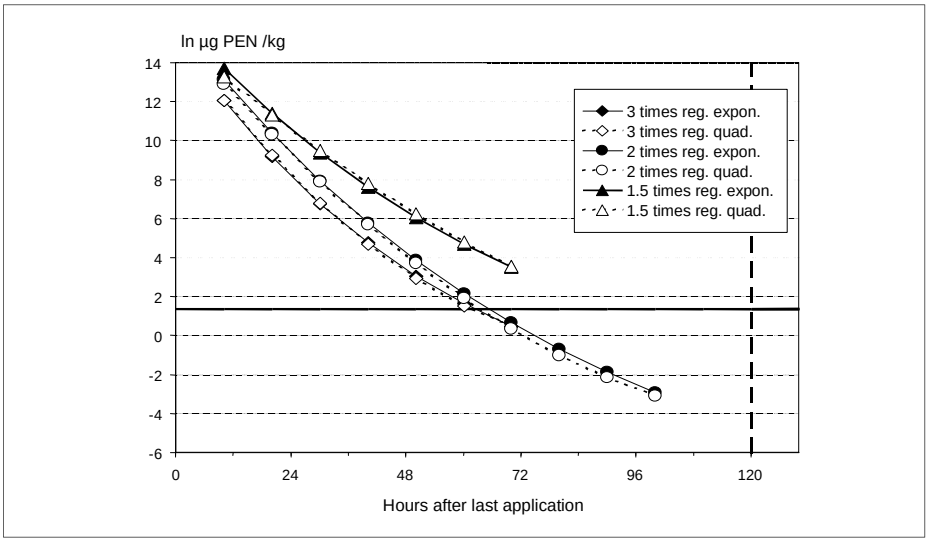

Fig. 8: Excretion of penicillin (Procain-Penicillin G 3 Mio. Euterinjektor) in milk in dependence on milking frequency – Quadratic and exponential regressions; ——— MRL, — — — Withholding time

Tab. 14: Withdrawal time of penicillin (in hours) in dependence on milking frequency – summarized results from pragmatic approach, TTSC and regression models; indicated withdrawal time: 120 h

Milking frequency	**3 times per day**		**2 times per day**		**1.5 times per day**	
Approach	Mean	95/95 tolerance limit[1]/ 95% confidence interval[2]	Mean	95/95 tolerance limit[1]/ 95% confidence interval[2]	Mean	95/95 tolerance limit[1]/ 95% confidence interval[2]
Pragmatic - individual	60		62		91	
Pragmatic - average	60		67		88	
TTSC[1]	64	93	68	96	95	158
Quadratic regression[2]	61	82	63	72	90	113
Exponent. regression[2]	62	78	64	73	92	120

[1] 95/95 tolerance limit [2] 95 % confidence interval

4.2.5 Analysis of variance

The model accounted for 84 % of the deviance of excretion time, but only the milking frequency had a significant influence on the withdrawal time. Using only the milking frequency 81 % of the variance were explained. The withdrawal time decreased with increasing milking frequency, but only the differences between milking 3 times per day and milking 1.5 times per day were significant (table 15).

Tab. 15: Withdrawal time (in hours) for penicillin (Least Square Means (LSQ_M) and standard error (se))

Milking frequency	LSQ_M ± se[1]
3 times	64.7 ± 4.6 [a]
2 times	65.7 ± 4.5 [ab]
1.5 times	96.3 ± 4.5 [b]

[1] Different superscripts show significant differences ($p<0.05$)

4.3 Nafpenzal® MC (Penicillin, Nafcillin, Dihydrostreptomycin)

4.3.1 Status of animals

The characterization of cows included in the experiments with the multicomponent drug Nafpenzal® MC is summarized in table 16.

Tab. 16: Nafpenzal® MC experiments – Status of animals

Cow	No. of lactation	Days in milk	Body weight (kg)	Milk yield per day (kg)[1]	SCC in composite milk (1000/ml)[1]	Mastitis pathogens
Milking frequency 3 times per day						
1751	5	84	684	31.5	56	LH: CNS
1831	2	140	767	27.3	51	LF: CNS
1842	2	101	732	35.1	17	n.d
1859	1	185	690	29.4	73	n.d
1880	1	173	534	25.2	56	RH: CNS
Milking frequency 2 times per day						
1818	3	185	733	26.4	53	n.d.
1827	2	176	660	23.8	45	n.d
1829	2	81	645	32.4	41	LF+RH: CNS
1846	2	110	652	30.6	51	n.d
1867	1	143	585	21.4	36	n.d
Milking frequency 1.5 times per day						
1786	4	104	698	30.9	36	n.d
1830	2	165	745	30.7	41	n.d
1855	1	186	676	35.6	46	LH: CNS
1863	1	231	724	26.9	66	LF: CNS
1870	1	215	595	26.6	42	LH: Coryneform

[1] average during experimental period, n.d. = not detected, CNS = coagulase-negative staphylococci; quarters: RF = right front, LF = left front, RH = right hind, LH = left hind

4.3.2 SCC

In figure 9 the SCC in cow composite milk samples is demonstrated as average per group.

4.3.3 Excretion

In table 17 the excreted quantities of PEN, NAF and DHS via milk are summarized per cow and group with respect to the milking interval.

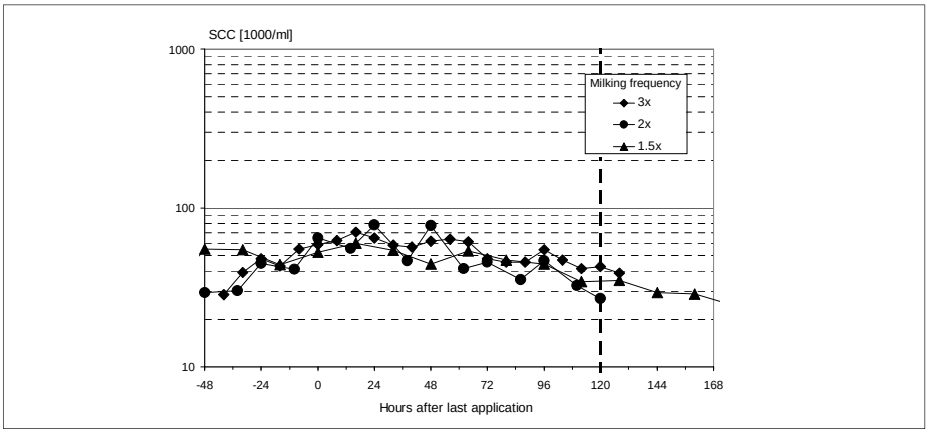

Fig. 9: SCC in milk in dependence on milking frequency - Nafpenzal® MC experiments – Average of 5 cows per group; — — — Withholding time

Tab. 17: Nafpenzal® MC experiments: penicillin, nafcillin and dihydrostreptomycin excretion via milk in dependence on milking interval

Cow no.	Milk yield (kg)	Excreted PEN mg	Excreted PEN in % of applied[1]	Excreted NAF mg	Excreted NAF in % of applied[1]	Excreted DHS mg	Excreted DHS in % of applied[1]
Milking frequency: 3 times per day							
1842	35.1	1 018	47.1	448	37.3	1097	91.4
1751	31.5	885	41.0	302	25.2	958	79.8
1859	29.4	1 068	49.4	494	41.2	1 065	88.8
1831	27.3	682	31.6	261	21.8	718	59.8
1880	25.2	988	45.7	448	37.3	957	79.8
Average	**29.7**	**928**	**43.0**	**391**	**32.6**	**959**	**79.9**
Milking frequency: 2 times per day							
1829	32.4	642	29.7	237	19.8	797	66.4
1846	30.6	960	44.4	394	32.8	975	81.3
1818	26.4	1 078	49.9	348	29.0	1 219	101.5
1827	23.8	844	39.1	313	26.1	979	81.5
1867	21.4	692	32.0	283	23.6	585	48.8
Average	**26.8**	**843**	**39.0**	**315**	**26.3**	**911**	**75.9**
Milking frequency: 1.5 times per day							
1855	35.6	371	17.2	162	13.5	429	37.8
1786	30.9	514	23.8	171	14.3	647	53.9
1830	30.7	613	28.4	227	18.9	658	54.8
1863	26.9	637	29.5	248	20.7	662	55.1
1870	26.6	643	29.8	265	22.0	693	57.8
Average	**30.1**	**556**	**25.7**	**215**	**17.9**	**618**	**51.5**

[1] 100 % = 2160 mg PEN, 1200 mg NAF, 1200 mg DHS

4.3.4 Withdrawal time

In figures 10 to 12 the excretion of PEN, NAF and DHS is demonstrated for the individual cows within each experimental group. The average curves are demonstrated in figure 13. The indicated withdrawal times differ with respect to the country where the drug is registered (table 3). In the graphs a withdrawal time of 120 hours as valid in the Netherlands is indicated. The quadratic and exponential regressions for each substance are presented in figure 14. Table 18 summarizes the results of the determination of withdrawal times by different approaches. The longest withdrawal periods are necessary for PEN whereas the shortest excretion period was observed for NAF.

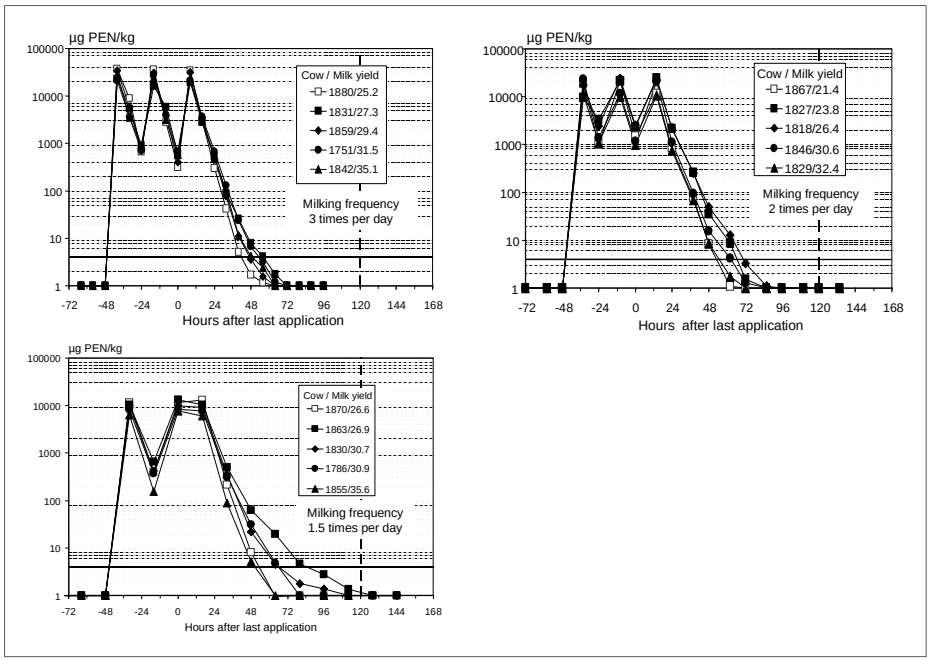

Fig. 10: Excretion of penicillin (Nafpenzal® MC) in milk of individual cows; ———— MRL — — — Withholding time

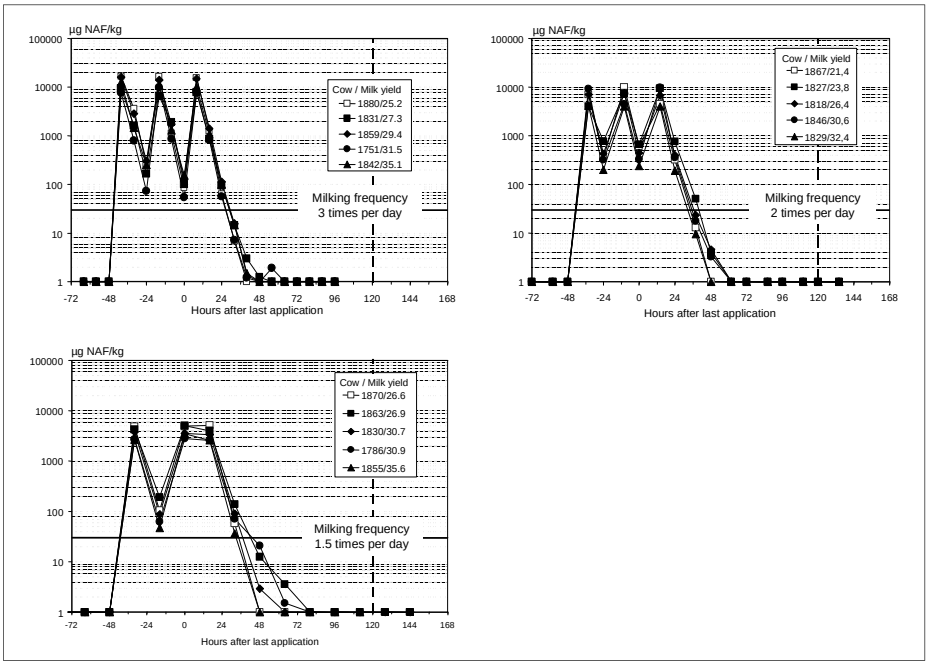

Fig. 11: Excretion of nafcillin (Nafpenzal® MC) in milk of individual cows; ——— MRL, — — — Withholding time

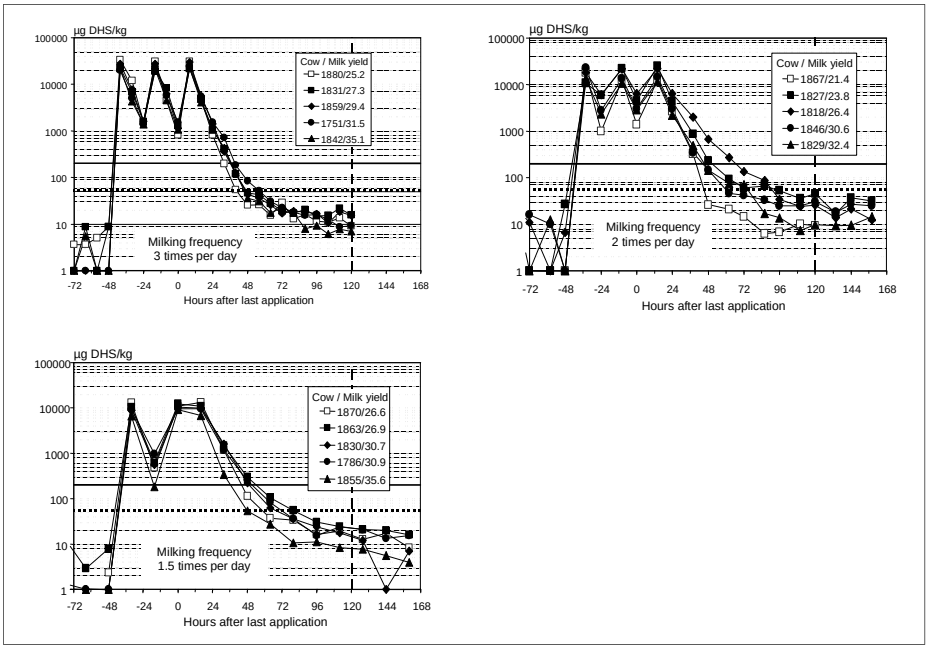

Fig. 12: Excretion of dihydrostreptomycin (Nafpenzal® MC) in milk of individual cows; ——— MRL, — — — Withholding time, ▪▪▪▪▪ Limit of quantification

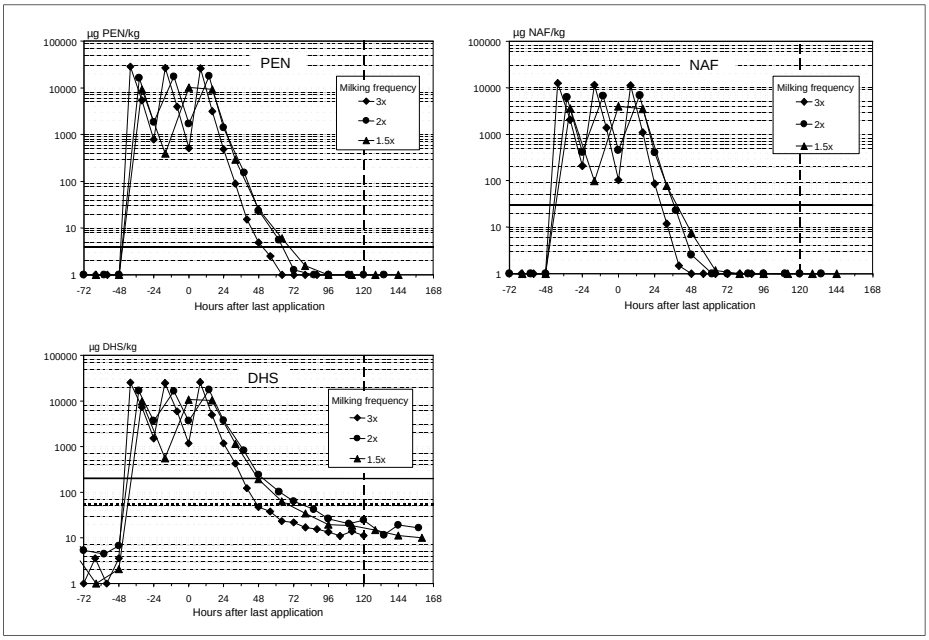

Fig. 13: Excretion of antibiotics (Nafpenzal® MC) in milk in dependence on milking frequency – Average of 5 cows per group; ——— MRL, — — — Withholding time, ■■■■■■ Limit of quantification

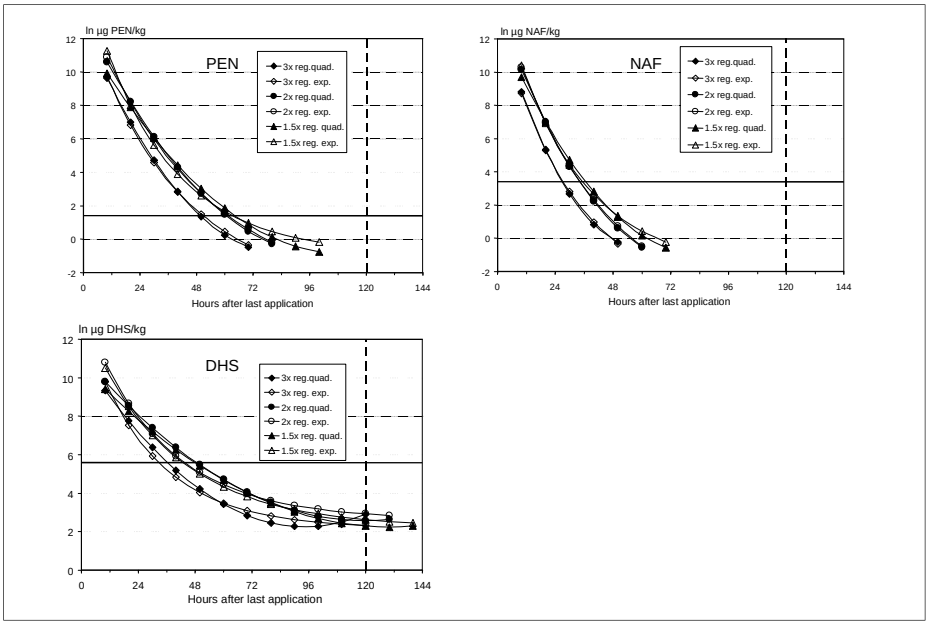

Fig. 14: Excretion of antibiotics (Nafpenzal® MC) in milk in dependence on milking frequency – Quadratic and exponential regressions; ——— MRL, — — — Withholding time

Tab. 18: Withdrawal time (in hours) of Nafpenzal® MC in dependence on milking frequency – summarized results from pragmatic approach, TTSC and regression model; indicated withdrawal time: UK 72 h, NL 120 h

Milking frequency	3 times per day		2 times per day		1.5 times per day	
Substance/ Approach	Mean	95/95 tolerance limit[1]/ 95% confidence interval[2]	Mean	95/95 tolerance limit[1]/ 95% confidence interval[2]	Mean	95/95 tolerance limit[1]/ 95% confidence interval[2]
PEN						
Pragmatic - individual	50		62		69	
Pragmatic - average	52		67		72	
TTSC[1]	54	90	68	96	76	157
Quadratic regression[2]	50	57	61	75	65	100
Exponent. regression[2]	51	61	62	80	64	120
NAF						
Pragmatic - individual	28		33		40	
Pragmatic - average	28		31		40	
TTSC[1]	32	32	40	62	48	48
Quadratic regression[2]	27	31	34	39	37	46
Exponent. regression[2]	27	34	34	41	35	48
DHS						
Pragmatic - individual	36		50		50	
Pragmatic - average	36		55		40	
TTSC[1]	38	58	58	122	57	111
Quadratic regression[2]	39	48	52	75	52	70
Exponent. regression[2]	35	42	47	73	46	61

[1] 95/95 tolerance limit, [2] 95 % confidence interval

4.3.5 Analysis of variance

The predicted withdrawal times for all three antibiotics are summarized in table 19. The analysis of variance was performed without the variance factor body weight as in the CEF and PEN experiments and in addition with the factor body weight.

For PEN the model accounted for 77 % of deviance of excretion time. Milking frequency ($p<0.01$) and lactation number ($p<0.05$) had significant influence on the withdrawal time. For milking frequency only the differences between the milking frequency 3 times per day and 1.5 times per day were significant. The predicted withholding period for cows in first lactation (56.1 hours) was shorter than for cows with lactation numbers >1 (70.1 hours). By including body weight only the milking frequency had significant influence on the excretion time. In this case the model explained 83 % of variance.

For NAF only milking frequency ($p<0.01$) showed a significant influence on the withdrawal time. Due to the low variation between the withdrawal times 91 % of the deviance of time could be explained by the model. This remained unchanged by including the factor body weight. Withdrawal time decreased with increasing milking frequency. Differences between all three milking frequencies were significant (table 19).

For DHS the milking frequency ($p<0.01$) and the lactation number ($p<0.05$) had a significant influence on the withdrawal time. The model accounted for 80 % of deviance in time. The withdrawal time decreased with increasing milking frequency (table 19). Only the differences between the milking frequency of 3 times per day and the other milking frequencies were significant. Including body weight the model explained 87 % of deviance of time, but only the milking frequency had a significant influence ($p<0.01$).

Tab. 19: Withdrawal time (in hours) for Nafpenzal® MC (Least Square Means (LSQ_M) and standard error (se))

Substance Milking frequency	PEN $LSQ_M \pm se^1$	NAF $LSQ_M \pm se^1$	DHS $LSQ_M \pm se^1$
Without variance factor body weight			
3 times	51.1 ± 4.5 [a]	31.8 ± 1.6 [a]	34.8 ± 3.9 [a]
2 times	61.4 ± 4.7 [ab]	38.8 ± 1.7 [b]	48.9 ± 4.0 [b]
1.5 times	76.7 ± 5.3 [b]	48.0 ± 1.9 [c]	56.7 ± 4.6 [b]
With variance factor body weight			
3 times	53.5 ± 4.4 [a]	31.6 ± 1.9 [a]	37.1 ± 3.6 [a]
2 times	65.8 ± 5.1 [ab]	38.5 ± 2.1 [b]	53.1 ± 4.2 [b]
1.5 times	77.7 ± 4.9 [b]	47.9 ± 2.1 [c]	57.6 ± 4.0 [b]

[1] Different superscripts within column show significant differences ($p<0.05$)

By including the factor body weight the calculated withdrawal times for all antibiotics (as LSQ_M) slightly increased, but this had no influence on significance of differences between milking frequencies.

4.4 Omnygram® (Colistin, Ampicillin)

4.4.1 Status of animals

Data characterizing the cows included in the Omnygram® experiments are listed in table 20.

4.4.2 SCC

In figure 15 the average SCC of cow composite milk is shown for the three trials.

Tab. 20: Omnygram® experiments – Status of animals

Cow	No. of lactation	Days in milk	Body weight (kg)	Milk yield per day (kg)[1]	SCC in composite milk (1000/ml)[1]	Mastitis pathogens
Milking frequency 3 times per day						
1796	4	186	690	27.6	14	n.d.
1828	3	137	720	30.6	8	n.d.
1892	1	199	655	31.8	32	n.d.
1900	1	201	640	28.8	23	n.d.
1912	1	79	578	35.1	22	n.d.
Milking frequency 2 times per day						
1818	4	181	793	31.0	13	n.d.
1867	2	126	700	27.6	51	LF: CNS
1871	2	119	644	23.8	8	n.d.
1889	1	169	707	23.0	59	RH: Coryneform
1915	1	105	625	25.4	10	n.d.
Milking frequency 1.5 times per day						
1827	3	197	740	22.5	77	n.d.
1833	3	275	800	23.1	177	n.d
1835	3	132	780	24.7	25	n.d
1888	1	168	680	27.7	66	n.d.
1896	1	105	693	25.1	48	n.d.

[1] average during experimental period; [2] average of anamnestic period; n.d. = not detected, CNS = coagulase-neg. staphylococci; quarters: RF = right front, LF = left front, RH = right hind, LH = left hind

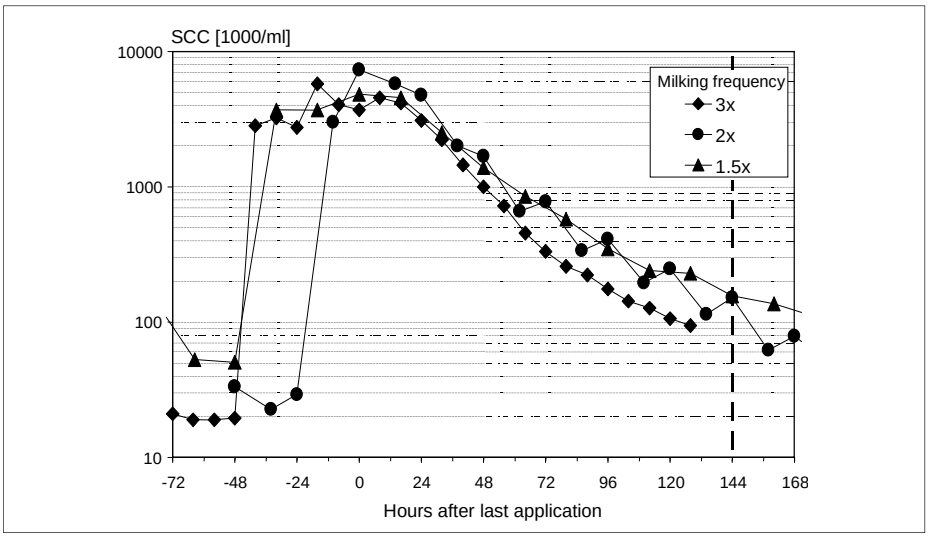

Fig. 15: SCC in milk in dependence on milking frequency - Omygram® experiments – Average of 5 cows per group; — — — Withholding time

4.4.3 Excretion

In table 21 the excreted quantities of AMP and COL via milk are summarized per cow and group with respect to the milking interval.

Tab. 21: Ampicillin and colistin excretion via milk in dependence on milking interval

Cow no.	Milk yield (kg)	Excreted AMP mg	Excreted AMP in % of applied[1]	Excreted COL mg	Excreted COL in % of applied[1]
Milking frequency: 3 times per day					
1796	27.6	812	7.8	480	48.5
1828	30.6	1 983	19.1	922	93.1
1892	31.8	943	9.1	520	52.5
1900	28.8	2 232	21.5	938	94.7
1912	35.1	1 133	10.9	402	40.6
Average	**30.8**	**1 421**	**13.7**	**652**	**65.9**
Milking frequency: 2 times per day					
1818	31.0	1 871	18.0	728	73.5
1867	27.6	1 348	13.0	663	67.0
1871	23.8	1 472	14.2	856	86.5
1889	23.0	1 238	11.9	454	45.9
1915	25.4	905	8.7	556	56.2
Average	**26.2**	**1 367**	**13.2**	**652**	**65.9**
Milking frequency: 1.5 times per day					
1827	22.5	482	4.6	759	76.7
1833	23.1	901	8.7	836	84.4
1835	24.7	108	1.0	263	26.6
1888	27.7	516	5.0	610	61.6
1896	25.1	846	8.1	761	76.9
Average	**24.6**	**570**	**5.5**	**646**	**65.3**

[1] 100 % = 10 392 mg AMP, 990 mg Colistin

4.4.4 Withdrawal time

The excretion curves of the individual cows within each experiment and the average curves are demonstrated in figures 16 to 18 for the two components (AMP, COL) of the applied drug. The quadratic and exponential regressions for each antibiotic with *ln* concentration scales are presented in figure 19.

The results of the different approaches to determine the withholding periods are summarized in table 22.

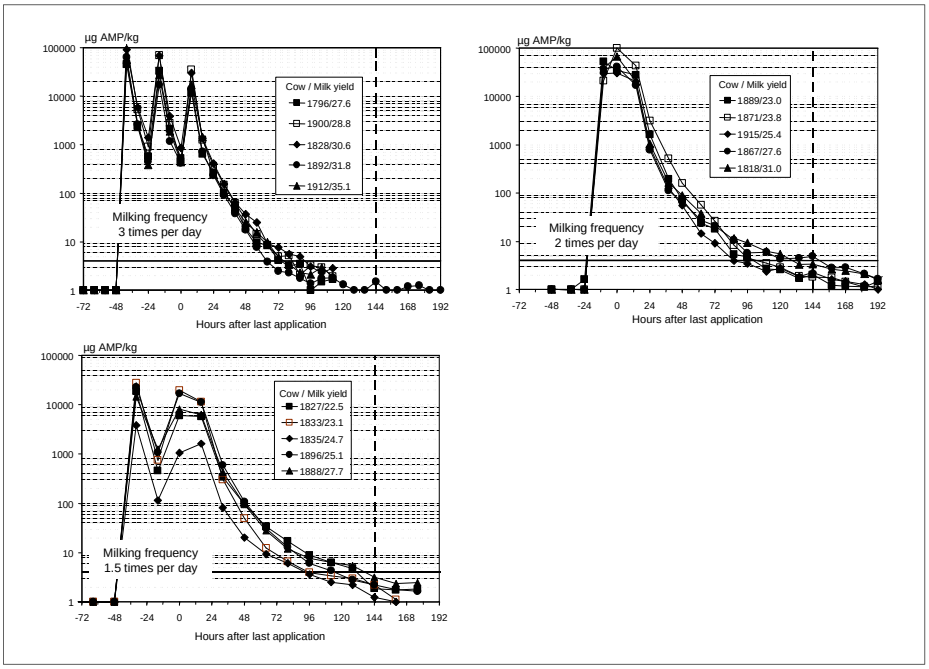

Fig. 16: Excretion of ampicillin (Omnygram®) in milk of individual cows; ———— MRL, — — — Withholding time

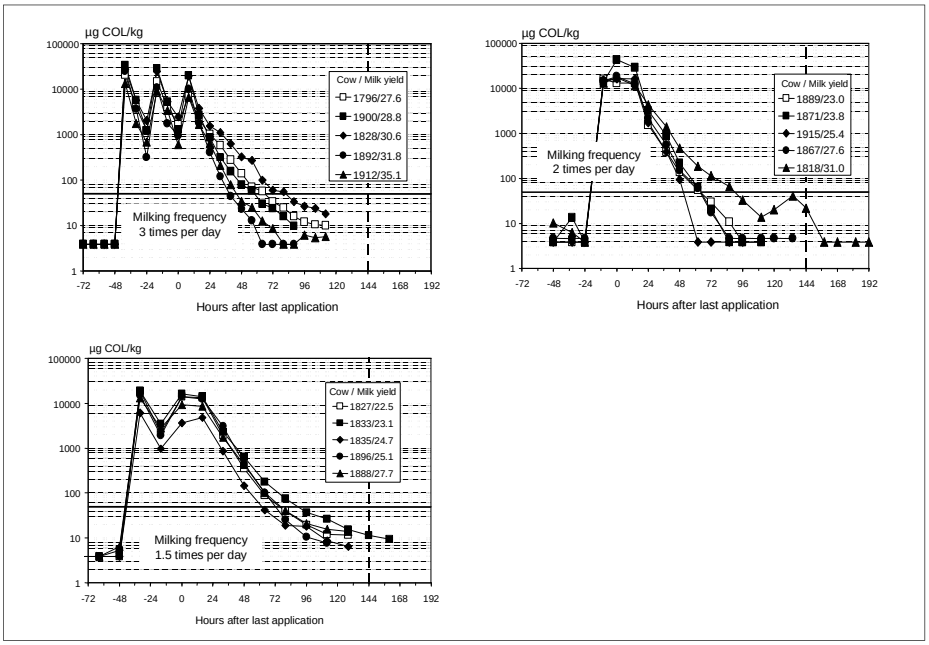

Fig. 17: Excretion of colistin (Omnygram®) in milk of individual cows; ———— MRL, — — — Withholding time

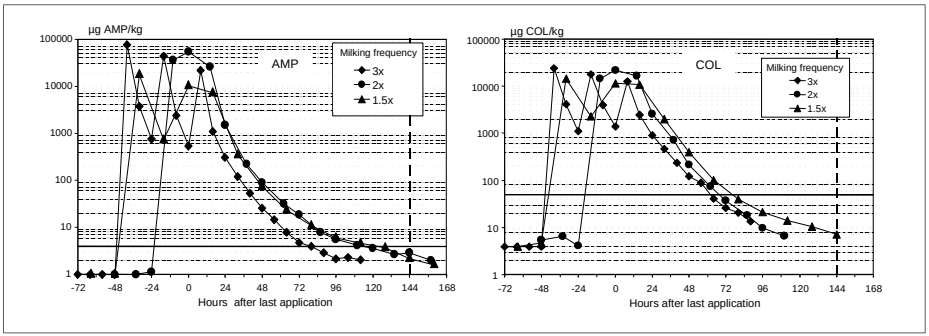

Fig. 18: Excretion of antibiotics (Omnygram®) in milk in dependence on milking frequency – Average of 5 cows per group; ——— MRL, — — — Withholding time

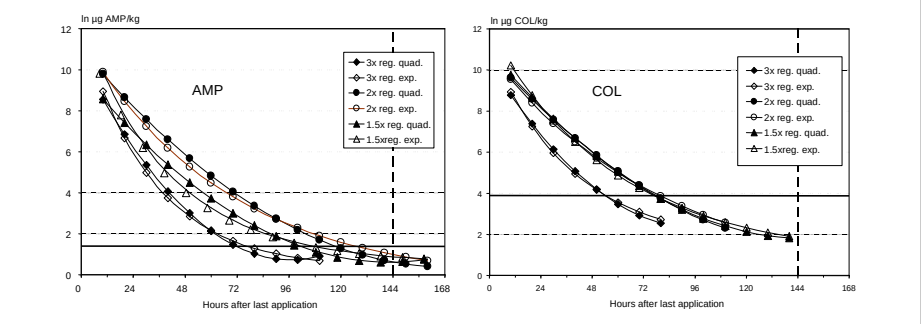

Fig. 19: Excretion of antibiotics (Omnygram®) in milk in dependence on milking frequency – Quadratic and exponential regressions; ——— MRL, — — — Withholding time

Tab. 22: Withdrawal time (in hours) of Omnygram® in dependence on milking frequency – summarized results from pragmatic approach, TTSC and regression model; indicated withdrawal time: 144 h

Milking frequency	**3 times per day**		**2 times per day**		**1.5 times per day**	
Substance/ Approach	Mean	95/95 tolerance limit[1]/ 95% confidence interval[2]	Mean	95/95 tolerance limit[1]/ 95% confidence interval[2]	Mean	95/95 tolerance limit[1]/ 95% confidence interval[2]
AMP						
Pragmatic - individual	78		125		116	
Pragmatic - average	76		127		120	
TTSC[1]	81	153	127	310	123	257
Quadratic regression[2]	72	*	118	*	102	*
Exponent. regression[2]	77	121	127	193	108	*
COL						
Pragmatic - individual	58		81		72	
Pragmatic - average	40		79		72	
TTSC[1]	68	219	89	194	92	168
Quadratic regression[2]	54	*	77	124	77	97
Exponent. regression[2]	54	105	79	142	76	99

[1] 95/95 tolerance limit, [b] 95 % confidence interval ; * due to the course of the curve the 95% confidence interval could not be derived

4.4.5 Analysis of variance

The experiment with milking 2 times per day was excluded from the analysis due to deviating treatment intervals. Therefore only milking frequencies of 3 times and 1.5 times per day were compared for this drug.

The results of the analysis of variance are summarized in table 23.

Tab. 23: Withdrawal time (in hours) for Omnygram® (Least Square Means (LSQ_M) and standard error (se))

Substance Milking frequency	AMP LSQ_M ± se [1]	COL LSQ_M ± se [1]
3 times	82.9 ± 27.8 [a]	62.3 ± 20.3 [a]
1.5 times	123.5 ± 8.5 [a]	69.5 ± 13.5 [a]

[1] Different superscripts within the same column show significant differences ($p<0.05$)

The model accounted for 68 % and 56 % of deviance of time for AMP and COL, respectively. For both antibiotics none of the variables had a significant influence on the withdrawal time. Including the factor body weight into the model only minor changes in the predicted values for the withholding time were produced (data not shown).

5. Discussion

5.1 Influence of milking frequency on excretion of antibiotics in milk

In table 24 the predicted withdrawal times from the model applied in the analysis of variance (see chapter 3.4) are summarized for all udder injectors used in the different experiments. For all four drugs applied - Cobactan® LC, Procain-Penicillin G 3 Mio. Euterinjektor, Nafpenzal® MC and Omnygram® – the milking frequency showed an influence on the excretion time of antibiotics in milk leading to shorter excretion periods with increasing milking frequency. Significant differences between certain experimental groups were observed for all veterinary drugs with the exception of Omnygram® containing the components AMP and COL. NAF was the only compound for which the excretion was significantly different for all three milking frequencies. For PEN (tested in two different drugs) only the excretion period of cows milked 3 times versus 1.5 times was significantly different. For CEF and DHS the excretion period for milking three times was significantly shorter compared to the other two groups, but there was no significant difference between milking 2 and 1.5 times.

These results are consistent with findings from Henschelchen and Walser (18) who observed shorter excretion periods in cows milked every two hours compared to cows milked two times per day after intramammary treatment with pencillin G and oxytetracycline, respectively. In these studies the applied amount was of minor importance for the duration of excretion.

For COL large variation in the excretion periods of individual cows was observed. In this case a higher number of animals would be needed to show significant differences. For AMP only a very small amount of the applied drug was excreted via milk. This may explain why the excretion is not significantly influenced by the milking frequency.

Tab. 24: Withdrawal time (in hours) for the different antibiotic drugs in healthy cows (Least Square Means (LSQ_M) and standard error (se))

	Milking frequency per day[1]		
	3 times	**2 times**	**1.5 times**
Cobactan® LC			
CEF	71.8 ± 6.5 [a]	111.6 ± 4.8 [b]	99.9 ± 5.6 [b]
Procain-Penicillin G 3 Mio. Euterinjektor			
PEN[2]	64.7 ± 4.6 [a]	65.7 ± 4.5 [ab]	96.3 ± 4.5 [b]
Nafpenzal® MC			
PEN[3]	51.1 ± 4.5 [a]	61.4 ± 4.7 [ab]	76.7 ± 5.3 [b]
NAF	31.8 ± 1.6 [a]	38.8 ± 1.7 [b]	48.0 ± 1.9 [c]
DHS	34.8 ± 3.9 [a]	48.9 ± 4.0 [b]	56.7 ± 4.6 [b]
Omnygram®			
AMP	82.9 ± 27.8 [a]	n.a.	123.5 ± 18.5 [a]
COL	62.3 ± 20.3 [a]	n.a.	69.5 ± 13.5 [a]

[1] Different superscripts within the same row show significant differences between milking frequencies ($p<0.05$)
[2] 1898 mg PEN per injector,
[3] 180 mg PEN per injector in a drug combination
n.a. = not applied

For most drugs the difference in excretion periods between cows milked 2 times and 1.5 times was smaller than between cows milked 2 times and 3 times per day, although the difference in time period is the same with 4 hours on average.

PEN was included in two treatment trials: first with injectors containing procain-PEN as single substance and second as a combination of PEN-Na with NAF and DHS. The single substance drug contained about 10 times the amount of PEN compared to the drug combination. For the drug with the higher concentration a longer excretion time in milk was observed, but this was most probably due to the sustained release of PEN from procain-PEN.

Additional factors with significant influence on the excretion time were the parameters days in milk (only for CEF in Cobactan® LC experiments) and number of lactation (PEN and DHS in Nafpenzal® MC-experiments). In the Nafpenzal® MC trials the significant influence of lactation number on withdrawal time of PEN and DHS was neutralized by including body weight in the analysis of variance, which shows that the effect of lactation number and body weight are related.

The other factors included like milk yield, body weight and SCC had no influence on the time of excretion. Additional parameters were included in a study by Seymour *et al*. (35) who found that only the applied drug had significant influence on the excretion time. None of the factors route of administration, case number (in cows treated repeatedly due to clinical mastitis), number of days treated, body weight, lactation number or milk yield had significant influence. Milking frequency was not included in that study.

5.2 Withholding periods

For all antibiotics none of the individual cows exceeded the MRL of antibiotic residues in milk after the end of the withholding period. In the Cobactan® LC trials, however, one cow in each group had residue concentrations at MRL level at the last milking of the

withholding period or one day later (figure 2). In the experiment with the mishandled injectors the MRL was exceeded more frequently. In one cow concentrations exceeding the MRL were still detected 240 hours after the last application.

The withholding periods were calculated by different approaches. If a tolerance limit and a confidence interval were included, the indicated withholding period was not always sufficient. In the EU the official method to determine withholding periods for milk after intramammary treatment is the TTSC-method published by the EMEA (12). The calculated withholding times according to the TTSC-method including the 95/95 tolerance limit are summarized in figure 20. Considering that only 5 animals were included per treatment group a high tolerance factor had to be applied which resulted in withholding periods of up to 211 hours (almost 9 days instead of 5) for CEF. Therefore within the procedures for drug approval a minimum number of 19 cows is recommended for excretion trials (12). For PEN the withholding period of 5 days (120 hours) would only be exceeded when the 95/95 tolerance limit for the TTSC-method is applied.

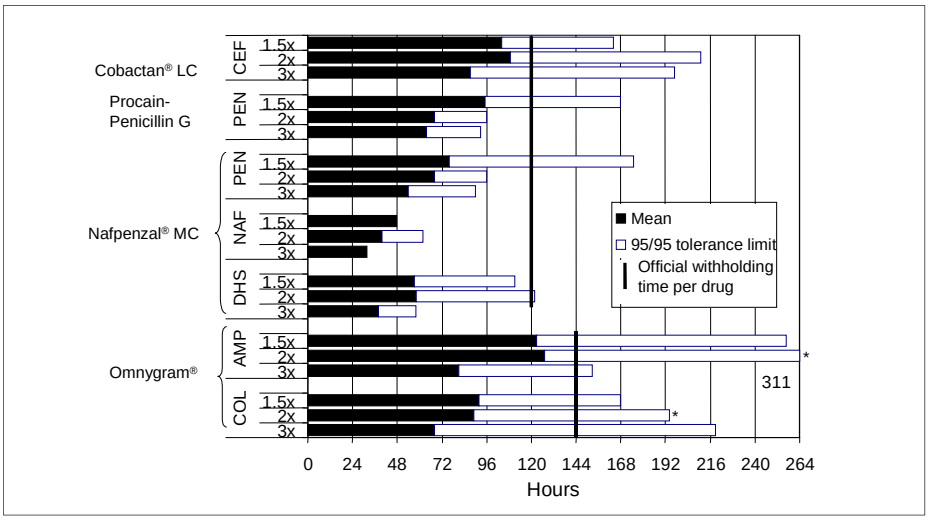

Fig. 20: Withholding times for antibiotics in four commercial drugs (intramammary application) calculated by TTSC-method in dependence on milking frequency; *deviating treatment scheme

The withholding period calculated by the TTSC approach resulted in the longest withholding time compared to the other approaches indicating a high level of consumer protection especially when the 95/95 tolerance limit is included.

Interpreting the results it has to be taken into account, that the experimental design is based on worst case conditions where the drugs were administered 3 times to all quarters of the cows. In practice, the probability of exceeding the MRL is expected to be lower, although in clinically diseased cows additional factors may affect the excretion time. These aspects are addressed in an ongoing experimental study.

5.3 Total amount of drug excreted via milk

The percentage of applied drug which was excreted via milk varied largely for the different antibiotics. The data are summarized in figure 21 in dependence on milking frequencies.

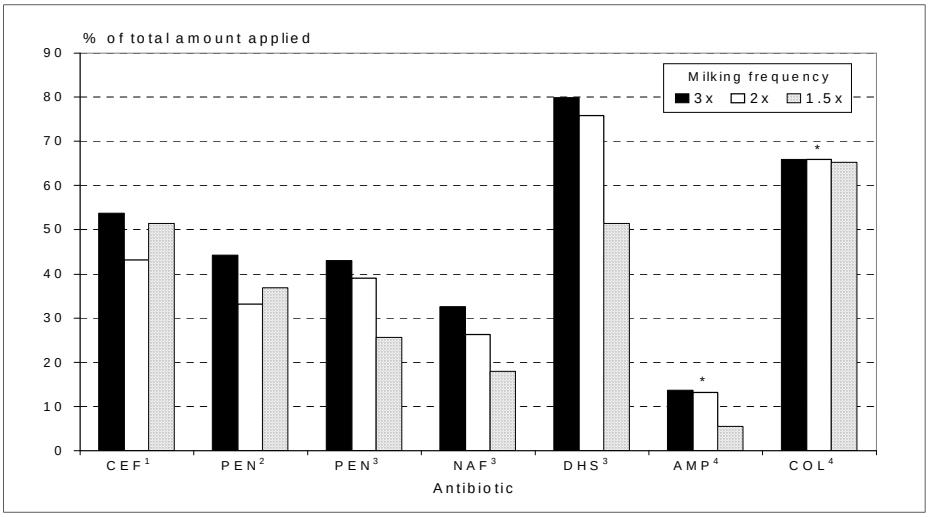

Fig. 21: Excreted amounts of antibiotic residues in milk in dependence on milking frequency
[1] Cobactan® LC; [2] Procain Penicillin G 3 Mio Euterinjektor, [3] Nafpenzal® MC, [4] Omnygram®, *deviating treatment scheme

For cows milked three times the percentage of antibiotic excreted via milk was in general higher than for cows milked less often. This can be interpreted in a way that more frequent milking leads to removal of antibiotics and after every milking a new balance of concentrations between tissue and newly secreted milk is formed. The effect was not observed with COL, a drug that is known to be of low lipid solubility and thus has difficulties to cross the blood milk barrier (49). The lowest amount of antibiotics excreted via milk was determined for AMP. The milk to serum concentration ratio for AMP is low (see table 1), which together with high lipid solubility leads to a diffusion from milk to serum. The highest amounts excreted via milk were observed for DHS, a polar substance which is in ionized form at physiological pH and shows low penetration of biological membranes. For COL the total percentage excreted via milk was nearly the same for all three groups (about 65 %), so drug release and removal via milk were independent from milking frequency. These findings can be explained by the physico-chemical properties of the drug resulting in limited ability to penetrate biological membranes. A higher proportion of the total amount of COL was expected to be excreted via milk because COL has similar properties as polymyxin B for which 90 % excretion via milk were observed by Ziv and Schultze (49).

For CEF and PEN as single substances the amount excreted via milk by cows milked 1.5 times was higher than for those milked 2 times per day. These differences are probably due to different excretion patterns in individual cows.

The interval between treatment and next milking influences the concentration of antibiotics in milk (figure 22). Higher concentrations in milk were observed when cows were milked in shorter intervals after application of the drug. This has to be regarded when cows are milked more frequently in AM systems. The comparison of two drugs containing PEN in different amounts showed that concentrations in milk were also higher after application of the higher dosage. Deviations from recommended dosages and treatment schemes (off-label use) may therefore influence the concentrations of antibiotics in milk as well as the duration of excretion in milk.

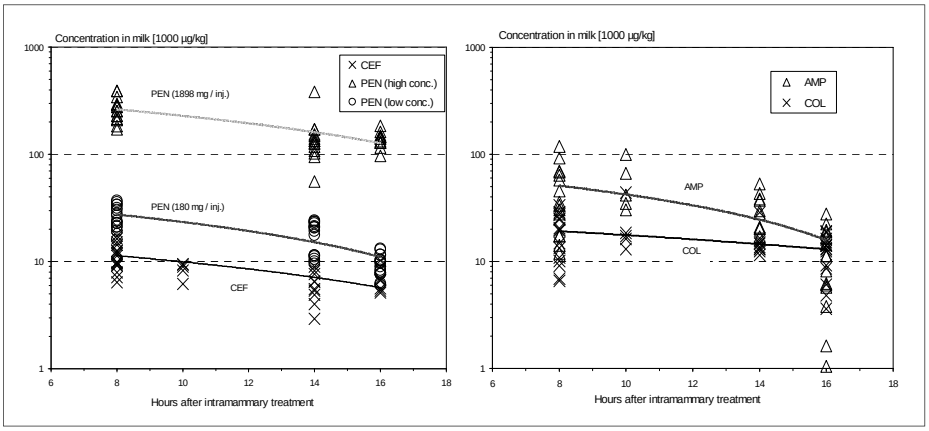

Fig. 22: Concentrations of antibiotics in milk in dependence on interval between treatment and milking

From these observations no conclusions can be drawn on possible influences of different milking frequencies on the efficacy of antibiotics in mastitis therapy, because concentrations in udder tissue were not determined.

5.4 Tissue reactions

The intramammary application of drugs did not lead to major rises in SCC in most treatment trials (see figures 1, 5 and 9). Only in the trials with Omnygram®, which contains COL, an antibiotic which is known to cause tissue irritations, cows reacted with a dramatic increase of SCC in milk (see figure 15). Even two days after the end of the withholding period the SCC did not return to the level of the anamnestic period. In milking times following an intramammary treatment all cows showed flakes in the milk for more than one milking time. Cows reacted with restlessness during milking and some kicked off the milking cluster. Considering these reactions in healthy cows it should be taken into account that in cows with clinical mastitis the time until SCC decreases to physiological levels could take much longer than the indicated withholding period for milk.

5.5 Recommendations of the manufacturer of drugs

Storage

In the first experiment with Cobactan® LC (CEF) the injectors used for the second of three intramammary administrations had inadvertently been stored at a temperature exceeding the storage temperature recommended by the manufacturer. This lead to a markedly prolonged excretion time in treated cows (figure 2 and 3). The injectors were subsequently analyzed by the manufacturer. The analysis showed noticeably changes in macroscopic aspects with changes in colour and homogeneity as well as an increased viscosity; the CEF content complied with the specification. Probably the changes in viscosity lead to retarded release of CEF from the formulations thus leading to the prolonged excretion period. The results clearly indicated that storage conditions deviating from those recommended by the manufacturer can lead to an extended excretion period.

Treatment intervals

Within the Omnygram® experiments different treatment schemes were applied for the trial with milking frequency of 2 times per day (3 treatments within 24 hours) compared to milking frequencies 3 and 1.5 times per day (3 treatments within 48 hours). Different treatment intervals were due to missing recommendations by the manufacturer. The recommended scheme of treatment during 3 successive milking times was only given on request. Unfortunately the two treatment trials with 3 and 1.5 times milking per day could not be repeated.

In the group milked two times per day treatment in shorter intervals lead to a higher concentration in milk after the third treatment than in the other two groups. This probably explains the slightly prolonged excretion time in this experiment which was unexpected. With treatment intervals comparable to the other two trials the excretion curve for this experiment most likely would be placed between the curves of the two other experiments.

6. Conclusions for users of AM systems

From the results of this study recommendations for users of AM systems can be derived, some of which also apply for farmers with conventional milking systems:

1. Farmers working with an AM system should ensure that treated cows are milked at least twice per day in regular intervals to make sure that the MRL in milk is not exceeded after the end of the withholding period. When dairy cows are treated with antibiotics the milking frequency has an effect on the excretion of antibiotics in the milk of individual cows leading to a longer excretion period with a reduced number of milkings per day. This effect is more or less pronounced for certain antibiotic drugs.
2. In any case the indicated withholding period has to be adhered to.
3. SCC in milk of individual quarters can be increased not only due to mastitis but also due to tissue reactions in response to treatment. Screening quarter milk samples for SCC is recommended before delivering milk to the bulk tank after the end of the withholding period.
4. If cow-side tests are applied to ensure that milk of individual cows is free of inhibitors after the end of the withholding period, farmers should apply screening tests with sufficient sensitivity to detect residues of the applied antibiotics at MRL concentrations.
5. Recommendations of the manufacturer regarding dosage, treatment interval and storage temperature should be followed closely to prevent prolonged drug excretion times in milk. Increased storage temperature lead to a significantly prolonged excretion period in milk in case of Cobactan® LC. For Omnygram® deviations from the expected drug excretion characteristics in milk were observed according to different treatment intervals.
6. In this study only the question of influence of different milking frequencies on excretion of antibiotic residues in milk was addressed. Therefore, conclusions cannot be drawn regarding the efficacy of mastitis treatment with antibiotics under these circumstances.

Acknowledgements

The work was funded by the European Commission within the EU project “Implications of the Introduction of Automatic Milking on Dairy Farms (QLK5-2000-31006)” as part of the EU-programme “Quality of Life and Management of Living Resources”.

The authors thank the milking personnel of the experimental station Schaedtbek of the Federal Research Centre for Nutrition and Food – location Kiel - for their involvement in performing the trials with different milking frequencies. Ernst-Heinrich Ubben is thanked for his support in determination of somatic cell count and Dr. Philipp Hammer for providing us with the antibodies for the ELISAs. The laboratory technicians, especially Heinz Braun and Maren Schmidt are gratefully acknowledged for excellent technical assistance.

Note

The content of this publication is the sole responsibility of its publisher, and does not necessarily represent the views of the European Commission nor any of the other partners of the project. Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use, which might be made of the information presented above.

7. References

(1) Andrade, J.R.A, Figueiredo, J.B., Lins, J.L.F.H.A.: Avaliação de liberação de resíduos de antibióticos no leite de vacas, pós medicação com penicillina e estreptomicina, em formulações aquosa e oleosa, coadjuvada pelo dimetilsulfóxido. Arquivo Brasileiro de Medicina Veterinaria e Zootecnica **45** 343-351 (1993)

(2) Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG: Suchverfahren auf das Vorhandensein von Antiinfektiva in Milch – Agar-Diffusionsverfahren mit Bacillus stearothermophilus (Brilliantschwarz-Reduktionstest); L01.00-11, February 1996

(3) Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG: Nachweis von Hemmstoffen in Milch – Agardiffusionsverfahren (Brilliantschwarz-Reduktionstest); L01.01-5, February 1996

(4) Cannon, R.Y., Hawkins, G.E., Wiggins, A.M.: Duration of secretion of bacteriostatic drugs in milk. I. Penicillin, following oral and parenteral administration. Journal of Dairy Science **45** 769-773 (1962)

(5) Council Directive 81/851 EEC of 28 September 1981 on the approximation of the laws of the Member States relating to veterinary medicinal products. Official Journal of the European Communities No. L 317, 06/11/1981

(6) Council Regulation (EEC) No. 2377/90 of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Official Journal of the European Communities No. L 224/1, 18/08/90, last modification by Regulation (EC) No. 6/2006, 06/01/06, Official Journal of the European Communities No. L 3/3

(7) Du Preez, J.H.: Treatment of various forms of bovine mastitis with consideration of udder pathology and the pharmacokinetics of appropriate drugs: a review. Journal of the South African Veterinary Association **59** 161-167 (1988)

(8) DVG Sachverständigenausschuss “Subklinische Mastitis”: Leitlinien zur Entnahme von Milchproben unter antiseptischen Bedingungen und Leitlinien zur Isolierung und Identifizierung von Mastitiserregern. Gießen, Germany, March 2000

(9) Edwards, S.J.: The diffusion and retention of penicillin after injection into the bovine udder. Veterinary Record **76** 545-549 (1964)

(10) Ehinger, A.M., Kietzmann, M.: Pharmakokinetische Aspekte der Mastitistherapie. Berliner und Münchner Tierärztliche Wochenschrift **111** 337-343 (1998)

(11) Erskine, R.J., Wilson, R.C., Tyler, J.W., McClure, K.A., Nelson, R.S., Spears, H.J.: Ceftiofur distribution in serum and milk from clinically normal cows and cows with experimentally *Escherichia coli*-induced mastitis. American Journal of Veterinary Research **56** 481-485 (1995)

(12) European Agency for the Evaluation of Medicinal Products (EMEA) - Committee for Veterinary Medicinal Products: Note for guidance for the determination of withdrawal periods for milk. EMEA/CVMP/473/98-final

(13) EMEA – Committee for Veterinary Medicinal Products: Summary Report – Penicillins.
(14) EMEA – Committee for Veterinary Medicinal Products: Summary Report – Cefquinome. EMEA/MRL/005/95
(15) Fabre, J.M., Moretain, J.P., Ascher, F., Brouillet, P., Bertelot, X.: Main causes of inhibitors in milk – a survey in one thousand French dairy farms. Proceedings of the Symposium on residues of antimicrobial drugs and other inhibitors in milk, August 28-31, 1995, Kiel, Germany, International Dairy Federation S.I. 9505, pp. 27-31 (1995), ISBN 92 9098 021 4
(16) Hammer, P.: ELISA zum Nachweis von Colistin aus Rohmilch. Tagungsbericht der 39. Arbeitstagung des Arbeitsgebietes Lebensmittelhygiene der Deutschen Veterinärmedizinischen Gesellschaft, e.V., September 22-25, 1998, Garmisch-Partenkirchen, Germany, pp. 411-414 (1998)
(17) Hammer, P., Kirchhoff, H., Hahn, G.: Detection of streptomycins in raw milk by an antibody-capture immunoassay. Analytica Chimica Acta **275** 313-316 (1993)
(18) Henschelchen, O., Walser, K.: Effect of milking interval on antibiotic residues in milk after intramammary treatment. Tierärztliche Umschau **838** 83-85 (1986)
(19) International Dairy Federation (IDF): Milk, Enumeration of somatic cells, Method C, Fluoro-opto-electronic method. IDF Standard 148A:1995
(20) International Standard Organisation(ISO)/International Dairy Federation (IDF): Milk and milk products – Guidelines for a standardized description of microbial inhibitor tests. ISO 13969/IDF 183 (2003)
(21) Kirchhoff, H.: Entwicklung eines enzymimmunologischen Nachweisverfahrens für Streptomycine in Milch. Dissertation med. vet., Freie Universität Berlin, Journal Nr. 1628 (1992)
(22) Koppinen, J., Heinonen, K., Heinonen, K.: Lihakseen annetun prokaiinipenisilliinin aiheuttamat jäämät hoidettujen lehmien maidossa. Suomen Eläinlääkärilehti **107** 235-239 (2001)
(23) Mäyrä-Mäkinen, A.: Technological significance of residues for the dairy industry. Proceedings of the Symposium on residues of antimicrobial drugs and other inhibitors in milk, August 28-31, 1995, Kiel, Germany, International Dairy Federation S.I. 9505, pp. 136-143 (1995), ISBN 92 9098 021 4
(24) Malvisi, J., Anfossi, P., Zaghini, A., Stracciari, G.L.: Presenza di ossitetraciclina, neomicina e oleandomicina nel siero e nel latte di bovine trattate per infusione endomammaria. Archivio Veterinario Italiano **42** 36-47 1991
(25) Moretain, J.P., Boisseau, J.: Excretion of penicillins and cephalexin in bovine milk following intramammary administration. Food Additives and Contaminants **6** 79-90 (1989)
(26) Mygind, J.: The Danish Industrial Association of Veterinary Medicine (VIF): Personal communication, November 2000
(27) Nouws, J.F.M., Ziv, G.: Distribution and residues of macrolide antibiotics in normal dairy cows. Archiv für Lebensmittelhygiene **30** 202-208 (1979)
(28) Rasmussen, M.D., Justesen, P.: The frequency of antibiotics in milk from herds with automatic milking systems. Italian Journal of Animal Science **2** 318 (2003)
(29) Roncada, P., Tomasi, L., Stracciari, G.L., Ermini, L., Strocchia, A.: Milk depletion of dicloxacillin residues in cows and sheep following intramammary administration. Journal of Veterinary Pharmacology and Therapeutics **23** 237-241 (2000)
(30) Rule, R., Quiroga, G., Buschiazzo, H., Lacchini, R., Mordujovich, P.: Rate of decline of cefotaxime and ceftazidime in milk following intramammary administration to healthy and mastitic dairy cows. Veterinary Record **143** 310-311 (1998)
(31) SAS Release 8.01. SAS Institute Inc., Cary, NC, USA
(32) Schällibaum, M.: Antibiotikatherapie und Rückstände in der Anlieferungsmilch. Swiss Vet **7** 7-9 (1990)
(33) Schällibaum, M., Nicolet, J., Bosson, J.: Die Behandlung von chronisch-subklinischen Staphylokokkenmastitiden beim laktierenden Rind durch die Verabreichung hoher Spiramycin-dosen. Schweizer Archiv für Tierheilkunde **123** 277-292 (1981)
(34) Schluep, J., Rosselet, A., Heim, H.: Comparative evaluation of cephacetrile in experimentally induced bovine mastitis. Zentralblatt für Veterinärmedizin B **26** 304-324 (1979)
(35) Seymour, E.H., Jones, G.M., McGilliard, M.L.: Persistence of residues in milk following antibiotic treatment of dairy cattle. Journal of Dairy Science **71** 2292-2296 (1988)

(36) Suhren, G.: Hemmstoffe und Tierarzneimittelrückstände in Milch – rechtliche Grundlagen, Nachweisverfahren, Untersuchungssysteme. Kieler Milchwirtschaftliche Forschungsberichte **54** 35-71 (2002)
(37) Suhren, G., Knappstein, K.: Detection of incurred dihydrostreptomycin residues in milk by liquid chromatography and preliminary confirmation methods. Analyst **23** 2797-2801 (1998)
(38) Suhren, G., Knappstein, K.: What do MRL levels of antibiotics mean with respect to technological safety? Congrilait – 26th IDF World Dairy Congress, September 24-27, 2002, Paris, France, Poster D1-05 (2002)
(39) Suhren, G., Knappstein, K.: Detection of cefquinome in milk by liquid chromatography and screening methods. Analytica Chimica Acta **483** 363-372 (2003)
(40) Suhren, G., Knappstein, K.: Detection of residues of antibiotics in milk of treated cows by screening methods. Milchwissenschaft **59** 656-659 (2004)
(41) Suhren, G., Knappstein, K.: Detection of colistin in spiked and incurred milk samples by LC- and ELISA-technique. Analytica Chimica Acta **529** 97-101 (2005)
(42) Suhren, G., Walte, H.-G.: Experiences with the application of method combinations for the detection of residues of antimicrobial drugs in milk from collecting tankers. Milchwissenschaft **58** 536-540 (2003)
(43) Suppliers of veterinary medicinal products in Germany, personal communication
(44) Vukovic, V., Kasalica, A.: Effect of penicillin in concentrations below the sensitivity level of biological methods on yoghurt cultures and yoghurt. Acta Alimentaria **33** 167-174 (2004)
(45) Walter, A.M., Heilmeyer, L.: Antibiotika Fibel. 4th Edition, Verlag Thieme Stuttgart (1975)
(46) Whittem, T.: Pharmacokinetics and milk discard times of pirlimycin after intramammary infusion: a population approach. Journal of Veterinary Pharmacology and Therapeutics **22** 41-51 (1999)
(47) World Health Organization (WHO): Principles for the safety assessment of food additives and contaminants in food. Environmental Health Criteria 70 (1987)
(48) Ziv, G. Pharmacokinetic concepts for systemic and intramammary antibiotic treatment in lactating and dry cows. Proceedings of the IDF Seminar on Mastitis Control, April 7-11, 1975, Reading, UK, pp. 314-340 (1975)
(49) Ziv, G., Schultze, W.D.: Pharmacokinetics of polymyxin B administered via the bovine mammary gland. Journal of Veterinary Pharmacology and Therapeutics **5** 123-129 (1982)
(50) Zurich, L.Z., San Martín, B.,N., Borie, C.P., Hermosilla, R.R.: Evolución de concentraciones de cefradina y cefoperazona en leche bovina normal y mastítica. Avances en Ciencias Veterinarias **8** 24-28 (1993)

8. Summary

Knappstein, K., Suhren, G., Walte, H.-G: **Influence of milking frequencies in automatic milking systems on excretion characteristics of different antibiotics in milk.** Kieler milchwirtschaftliche Forschungsberichte **57** (4) 215-261 (2005)

06 Automatic milking (Antibiotic residues, milking frequency, milk quality)

The prevention of antibiotic residues is an important aspect in milk production to ensure health protection for the consumer and to avoid failures during milk processing. Limited information is available on the potential impact of milking frequencies associated with Automatic Milking (AM) on excretion of veterinary drugs in milk.

Under experimental conditions the excretion of antibiotics in milk was studied in healthy cows (somatic cell count in cow composite milk below 100 000/ml) after intramammary treatment with 4 different commercially available drugs. The experimental groups were milked three times per day (milking interval 8 hours) or 1.5 times per day (milking interval of 16 hours). The excretion of antibiotics was compared to a reference group milked 2 times per day (milking interval 10 and 14 hours). The applied drugs contained 6 different

antibiotics as single substance or in combination. Residues of antibiotics in milk were determined by appropriate screening methods and HPLC-methods in cow composite milk sampled at every milking.

Different approaches for the determination of withholding periods were applied. For drugs containing cefquinome or penicillin or a combination of penicillin, nafcillin and dihydrostreptomycin significantly ($p<0.05$) shorter excretion periods were observed in cows milked 3 times per day compared to cows milked 1.5 times per day. For one drug containing ampicillin and colistin the differences were not significant although the same tendency was observed. Higher concentrations of residues in milk were determined with shorter intervals between treatment and next milking.

Milk yield, somatic cell count and days in milk had no significant influence on the excretion time of any antibiotic substance in milk. The lactation number influenced the excretion time of cefquinome as well as of penicillin and dihydrostreptomycin in a combined drug, but for the latter two antibiotics this effect was related to body weight.

Commercially available microbial inhibitor tests with *Bacillus stearothermophilus* as test microorganism were applied as screening methods and detected residues of penicillin G, ampicillin and nafcillin at concentrations $\leq$ Maximum Residue Limits (MRL), whereas sensitivities for the detection of cefquinome, dihydrostreptomycin and colistin were insufficient. As sensitive screening methods for the latter compounds ELISAs were applied.

Zusammenfassung

Knappstein, K., Suhren, G., Walte, H.-G: **Einfluss von Melkfrequenzen in automatischen Melkverfahren auf die Ausscheidungscharakteristika verschiedener Antibiotika in Milch.** Kieler milchwirtschaftliche Forschungsberichte **57** (4) 215-261 (2005)

06 Automatisches Melken (Antibiotika-Rückstände, Melkfrequenz, Milchqualität)

In der Milchgewinnung ist die Vermeidung von Antibiotika-Rückständen ein wichtiger Aspekt zur Sicherstellung des gesundheitlichen Verbraucherschutzes und zur Vermeidung von Produktionsfehlern bei der Herstellung von Milchprodukten. Über den möglichen Einfluss von Änderungen der Melkfrequenz im Zusammenhang mit dem Einsatz von automatischen Melksystemen auf die Antibiotika-Ausscheidung in Milch liegen nur sehr begrenzte Informationen vor. Unter experimentellen Bedingungen wurde daher die Ausscheidung von Antibiotika in Milch nach intramammärer Behandlung von euergesunden laktierenden Kühen (somatischer Zellgehalt im Gesamtgemelk unter 100 000/ml) mit vier kommerziell erhältlichen Euterinjektoren untersucht. Die Versuchsgruppen wurden 3-mal täglich (Melkintervall 8 Stunden) bzw. 1,5-mal täglich (Melkintervall 16 Stunden) gemolken und die Antibiotika-Ausscheidung mit der einer Referenzgruppe, die 2-mal täglich (Melkintervall 10 und 14 Stunden) gemolken wurde, verglichen. Die eingesetzten Euterinjektoren enthielten sechs verschiedene Antibiotika allein oder in Kombination. Die Rückstände in zu jeder Melkzeit entnommenen Gesamtgemelken wurden mit geeigneten Screening- und HPLC-Methoden bestimmt.

Zur Berechnung der Wartezeiten wurden verschiedene Verfahren angewendet. Bei Arzneimitteln, die Cefquinom, Penicillin oder eine Kombination aus Penicillin, Nafcillin und Dihydrostreptomycin enthielten, wurden bei 3-mal täglich gemolkenen Kühen signifikant kürzere Ausscheidungszeiten ermittelt als bei 1,5-mal täglichem Melken

(p<0,05). Für ein Kombinationspräparat aus Ampicillin und Colistin waren die Unterschiede nicht signifikant, obwohl eine ähnliche Tendenz beobachtet wurde. Bei kürzeren Intervallen zwischen Behandlung und nächstem Melken wurden höhere Rückstandskonzentrationen in Milch gefunden.

Milchmenge, somatischer Zellgehalt in Milch und Laktationsdauer zeigten keinen signifikanten Einfluss auf die Ausscheidungsdauer der einbezogenen Antibiotika. Bei Cefquinom sowie bei Penicillin und Dihydrostreptomycin in einem Kombinationspräparat zeigte die Laktationsnummer einen Einfluss auf die Ausscheidungsdauer, bei den beiden letzteren Substanzen war dieser Effekt jedoch mit dem Körpergewicht assoziiert.

Kommerziell erhältliche mikrobiologische Hemmstofftests mit *Bacillus stearothermophilus* als Testmikroorganismus waren geeignet, um im Screeningverfahren Penicillin G, Ampicillin und Nafcillin in Konzentrationen ≤ MRL (Maximum Residue Limit) nachzuweisen. Die Nachweisempfindlichkeiten für Cefquinom, Dihydrostreptomycin und Colistin waren dagegen unzureichend. Als empfindliche Screeningverfahren für die beiden letzteren Substanzen wurden ELISAs eingesetzt.

Résumé

Knappstein, K., Suhren, G. Walte, H.-G: **Influence des fréquences de traite dans des systèmes de traite automatique sur les caractéristiques d'excrétion de différents antibiotiques dans le lait.** Kieler Milchwirtschaftliche Forschungsberichte **57** (4) 215-261 (2005)

06 Traite automatique (résidus antibiotiques, fréquence des traites, qualité du lait)

Dans la production du lait, la prévention de résidus antibiotiques est importante pour protéger la santé des consommateurs et pour éviter des erreurs dans la fabrication des produits laitiers. On a peu d'informations sur l'influence potentielle des fréquences de traite sur l'excrétion d'antibiotiques dans le lait en ce qui concerne la traite automatique. Par conséquent, on a analysé l'excrétion d'antibiotiques dans le lait sous des conditions expérimentales dans des vaches lactantes à mamelles saines (teneur en cellules somatiques dans la traite complète en-dessous de 100 000/ml) après un traitement intramammaire avec 4 médicaments différents offerts sur le marché. Les vaches des groupes expérimentaux étaient traites 3 fois par jour (intervalle de traite : 8 heures) respectivement 1,5 fois par jour (intervalle de traite : 16 heures). L'excrétion d'antibiotiques était comparée à celle d'un groupe de référence trait 2 fois par jour (intervalle de traite : 10 et 14 heures). Les injecteurs mammaires contenaient six différents antibiotiques, soit sous forme solitaire ou combinée. Les résidus dans les traites complètes prélevés à chaque traite étaient déterminés par des méthodes appropriées de screening et de CLHP.

Différentes méthodes étaient appliquées pour calculer les temps d'attente. Pour des médicaments contenant de la cefquinome, pénicilline ou une combinaison de pénicilline, nafcilline et dihydrostreptomycine, des temps d'excrétion nettement plus courts étaient enregistrés pour des vaches traites 3 fois par jour que pour des vaches traites 1,5- fois par jour (p<0,05). Avec un médicament composé d'ampicilline et de colistine, les différences étaient insignifiantes, néanmoins une tendance similaire était observée. Des concentrations de résidus plus élevées étaient enregistrées avec des intervalles plus courts entre le traitement et la prochaine traite.

Quantité de lait, teneur en cellules somatiques et durée de lactation n'avaient pas d'influence signifiante sur la durée d'excrétion des antibiotiques concernés. Pour la cefquinome, pénicilline et dihydrostreptomycine dans un médicament composé, le numéro de lactation exerçait une influence sur la durée d'excrétion. Pour les deux dernières substances pénicilline et dihydrostreptomycine, cet effet était associé au poids du corps.

Des tests d'inhibitions microbiologiques offerts sur le marché, contenant *Bacillus stearothermophilus* comme organisme de contrôle, étaient appropriés pour détecter les substances pénicilline G, ampicilline et nafcilline dans des concentrations = MRL (Limite Maximale de Résidus) dans la méthode screening. Les sensibilités de détection pour cefquinome, dihydrostreptomycine et colistine étaient insuffisantes. Par conséquent, on a appliqué les méthodes de screening sensibles ELISA pour les substances sus-mentionnées.