Impact of Automatic Milking on Excretion of Antibiotic Residues
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Abstract
Prevention of antibiotic residues in milk is an important aspect in milk production to ensure health protection for the consumer and high quality of milk for dairy 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 composite milk below 100,000/ml) after intramammary treatment with 4 different commercially available drugs at milking frequencies of 3, 2 and 1.5 times per day. The concentrations of antibiotic residues in cow composite milk sampled at every milking time were determined by HPLC methods and compared to the Maximum Residue Limits (MRL) for each compound. 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 differences were not significant. Higher concentrations of residues in milk were determined with shorter intervals between treatment and next milking.

After intramammary treatment of clinical mastitis with cefquinome no significant difference of excretion times was detected between cows milked 2 times (n=15) or 1.5 times (n=4) per day. In these cows only milk yield had a significant influence on excretion time with shorter excretion times in high yielding cows (p<0.05). The indicated withholding periods for milk were sufficient for all drugs. Nevertheless it is recommended to milk treated cows at least twice per day, because prolonged excretion was determined in healthy cows milked less frequently.

1. Introduction
Prevention of antibiotic residues in milk is necessary to ensure health protection of consumers and to avoid failures during milk processing. In practice residues of antibiotics in milk are most often determined by microbial inhibitor tests. Treatment of mastitis with antibiotics is one of the most important causes associated with positive results of inhibitor tests on bulk tank milk (Fabre et al., 1995). In conventional milking management factors like accidental milking of treated cows, not attending the withholding time and failures during milking are the main reasons for antibiotic residues in bulk milk together with insufficient cleaning of milking equipment (Schällibaum, 1990). Rasmussen et al. (2003) reported a higher risk for inhibitor positive tests to be associated with Automatic Milking (AM). Like in conventional milking management failures were the main reasons for residues in milk.

Little is known about the impact of milking intervals on the excretion of antibiotic residues in milk. Previous investigations were mainly focused on the effect of stripping on concentrations of antibiotics in milk after treatment of mastitis. Schluep and Heim (1980) found no difference for cefacetetrile excretion in quarters stripped 4 times within 10 hours between treatment and next regular milking compared to longer intervals in quarters of the same cow. In contrast, Henschelchen and Walser (1983) determined significantly shorter excretion periods for procain-penicillin G and oxytetracycline...
when cows were stripped in 2-hour intervals after treatment. Both groups made their investigations with healthy cows.

The distribution of antibiotics in the udder is dependent on the vehicle used in drugs for intramammary infusions as well as on the physico-chemical properties of the antibiotic. High molecular weight, low lipid solubility and high degree of ionization at pH of milk prevent antibiotics from permeation of the blood milk barrier after intramammary treatment (Ziv, 1975). The distribution of antibiotics changes due to increased pH of milk in quarters with acute clinical mastitis.

The aim of the study reported here was to determine the excretion of antibiotics in milk in dependence on milking frequency. Four drugs were studied in healthy cows milked with three different milking frequencies. One drug was selected for further analysis in cows with clinical mastitis. The concentration of residues in milk at the end of the withholding period was compared to Maximum Residue Limits (MRL) set by Council Regulation 2377/90/EEC.

2. Materials and Methods

2.1 Excretion trials

Excretion trials were performed under experimental conditions simulating milking frequencies as observed in AM systems.

2.1.1 Dairy cows

Trials in healthy cows

German Holstein, groups of 5 (4 to 6) cows, lactation number 1 to 6, days after calving 41-299, somatic cell count (SCC) in composite milk <100 000/ml at three samplings in weekly intervals before beginning of treatment trial (in the following referred to as healthy cows), average milk yield per cow 21.4-37.8 kg per day, comparable milk yield between three groups tested with one drug, body weight 534-800 kg.

Trials in cows with clinical mastitis

German Holstein, groups of 12 and 4 cows, lactation number 1 to 5, days after calving 1-422, SCC in composite milk between 3.2 x 10^5/ml and 7.0 x 10^6/ml, average milk yield per day between 10.3 and 37.9 kg, body weight 610-851 kg. The number of clinical cases of mastitis per cow was: one case (7 cows), 2 cases (5 cows), 3 cases (1 cow); in 2 cows 2 quarters were affected at the same time. Infections with the following pathogens were determined: Staphylococcus aureus (2 quarters), coagulase-negative staphylococci (3 quarters), Streptococcus uberis (5 quarters), Escherichia coli/colliform bacteria (5 quarters), enterococci (2 quarters), coryneform bacteria (2 quarters) and mixed infections (2 quarters), no pathogen detected in 3 quarters.

2.1.2 Treatment

Drugs

The following drugs were used for intramammary treatment of healthy cows:

- Cobactan® LC (Hoechst Roussel Vet, now Intervet Int., Unterschleissheim, DE), 75 mg cefquinome (CEF) per injector (as CEF-sulfate), withholding time for milk: 5 days, MRL in milk: 20 µg/kg
- Procain-Penicillin G 3 Mio. (WDT, Garbsen, DE), 1898 mg Penicillin G (PEN) per injector (as procain-benzyl-PEN), withholding time for milk: 5 days, MRL in milk: 4 µg/kg
- Nafpenzal® MC (Intervet Int, Boxmeer, NL), 180 mg penicillin, 100 mg nafcillin and 100 mg dihydrostreptomycin (DHS) per injector (as PEN-sodium, NAF-
sodium and DHS-sulfate), withholding time for milk: 5 days, MRL in milk: PEN – 4 µg/kg, NAF - 30 µg/kg, DHS - 200 µg/kg

- Omnygram® (Virbac S.A., Carros, F), 866 mg ampicillin (AMP) and 82.5 mg colistin (COL) per injector (as AMP-trihydrate and COL-sulfate), withholding time for milk: 6 days, MRL in milk: AMP - 4 µg/kg, COL - 50 µg/kg

Only Cobactan® LC was used for the excretion studies in cows with clinical mastitis.

**Treatment**

Healthy cows: 4 udder quarters per cow (worst case), one injector per quarter, 3 treatments within 24 hours (Cobactan® LC and Omnygram® - milking 2x/day) or 48 hours (Procain-Penicillin G 3 Mio., Nafpenzal® MC and Omnygram® - milking 3x and 1.5x/day), respectively. Milking times during the treatment period were adjusted to allow application of drugs in similar intervals for the 3 groups of cows tested per drug.

Cows with clinical mastitis: Treatment of quarters with clinical symptoms of disease, one injector Cobactan® LC per quarter, 3 treatments within 24 hours; additional treatment of one cow with 26 ml Cobactan® 2.5 % i.m. equiv. to 650 mg cefquinome

**Milking frequency**

Healthy cows: Per drug 3 groups of 5 cows tested with milking frequencies: 2 times per day (interval 14/10 hours, reference), 3 times per day (interval 8 h), 1.5 times per day (interval 16 h)

Cows with clinical mastitis: 2 times per day (17 cases), 1.5 times per day (5 cases)

**Sampling**

The experimental period included anamnesis (3 milkings or one milking for healthy and mastitic cows resp.), treatment period, indicated withholding period for milk plus 2 days. Sampling was extended if residues were detected more than 2 days after the end of the withholding period.

- quarter milk samples: for determination of somatic cell count (SCC) - at every milking; for determination of mastitis pathogens - healthy cows: one milking before start of treatment, cows with clinical mastitis: at every milking
- composite milk: for determination of SCC and residues of antibiotics, taken at every milking during the experimental period

**Storage of samples**

Max. 60 h at 6 °C; composite milk - max. 3 weeks at -20 °C for further investigations; lyophilisation and storage at 6 °C for later re-examinations if necessary

2.2 Laboratory analysis

2.2.1 Udder health

SCC was determined according to IDF Standard 148A:1995. Mastitis pathogens were identified according to the guidelines of the German Veterinary Association (DVG, 2000).

2.2.2 Detection of residues of antibiotics

*Qualitative detection*

Screening tests (microbial inhibitor tests, receptor assay, ELISAs) with sufficient sensitivities to detect antibiotic residues in milk at MRL concentrations were applied (Report D11, Knappstein et al., 2003).
Quantitative detection
For quantitative detection of residues HPLC methods were applied (Suhren and Knappstein, 1998, 2003, Suhren and Walte, 2003).

2.3 Determination of withdrawal time
The withdrawal time was determined by the Time-to-Safe-Concentration (TTSC) method according to the guidance of the European Agency for the Evaluation of Medicinal Products for the determination of withdrawal periods for milk (EMEA, 1998). Cows with clinical mastitis who received treatment on 2 quarters or additional parenteral treatment were excluded from calculations.

2.4 Analysis of variance
In order to determine which factors have a systematic influence on the withdrawal time an analysis of variance was carried out by use of the GLM (General Linear Model) procedure of SAS, release 8.01. The linear model had the following equation:

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

where \(Y_{ijkl}\) is the dependent variable (first time in hours when the antibiotic content fell below the MRL), \(\mu\) the overall mean, \(mf_i\) the effect of the \(i^{th}\) milking frequency (3, 2, 1.5), \(dac_j\) the effect of \(j^{th}\) days after calving (healthy cows: <100 d, >100 d; cows with clinical mastitis: <60 d, >60 d), \(ln_k\) the effect of the \(k^{th}\) number of lactation (1, >1). Milk yield and SCC as continuous variables were used as covariate with \(b_1\) the slope for milk yield, \(b_2\) the slope for SCC; \(e_{ijkl}\) the random residual error. In addition, body weight (kg) was included as influencing factor for Nafpenzal\(^{\circledR}\) MC and Omnygram\(^{\circledR}\) experiments as well as for Cobactan\(^{\circledR}\) LC trials in cows with clinical mastitis.

3. Results and Discussion
3.1 Excretion of residues in healthy cows
The excreted amount of residues in milk in percent of the total amount applied is summarized for all 4 drugs in dependence on milking frequency in figure 1.

Figure 1: Excreted amount of antibiotic residues in milk in % of total amount applied in dependence on milking frequency; 1 Cobactan\(^{\circledR}\) LC, 2 Procain-Penicillin G 3 Mio, 3 Nafpenzal\(^{\circledR}\) MC, 4 Omnygram\(^{\circledR}\), * deviating treatment scheme
Highest amounts of all drugs were excreted via milk in cows milked 3 times per day. No general tendency between milking frequency and excreted amount could be derived. In accordance with the pharmaco-kinetic properties of AMP the amount excreted via milk was very low. In contrast, high amounts of DHS (up to 80 %) and COL (65 %) were excreted via milk, which is probably due to the low lipid solubility of both drugs. For COL almost no difference was observed between the 3 groups of cows.

In figure 2 examples for the excretion of residues in milk of individual cows due to different milking frequencies are presented.

![Figure 2: Excretion of PEN in milk of individual cows after intramammary treatment in dependence on milking frequency](image)

Although the concentrations of PEN excreted in milk were similar for all cows at the beginning of treatment, more variation was seen after several milkings. Whereas the concentration was below the MRL after 72 hours in all cows milked 3 times per day, this was only the case after 112 h in cows milked with intervals of 16 hours. No relation was observed between milk yield and excretion time.

The influence of interval between treatment and next milking on the concentration in milk is shown for selected antibiotics in figure 3.

![Figure 3: Concentrations of different antibiotics in milk in dependence on interval between treatment and milking](image)
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 concentrations showed that concentrations in milk also increased with higher dosage of treatment. Deviations from recommended treatment schemes (off-label use) may therefore influence concentrations of antibiotics in milk as well as duration of excretion in milk.

The calculated withholding times according to the TTSC method are summarized for all four drugs in figure 4.

![Figure 4](image_url)

Figure 4: Withholding times for antibiotics in 4 commercial drugs (intramammary application) calculated by TTSC method in dependence on milking frequency; * deviating treatment scheme.

Withholding periods tended to be shorter with increasing milking frequency. The mean withholding periods were in accordance with the indicated withholding periods set for the respective drugs. If the 95/95 tolerance limit was calculated, in several cases the indicated withholding period was not sufficient, due to the large variation between individual cows and high safety factors. The reason for the high safety factor is the low number of cows (4 to 6 cows per group). To reduce the safety factor the use of a minimum number of 19 cows is recommended by EMEA (1998) for determination of withholding times. For PEN-treated cows milked 2 and 3 times per day and for NAF- and DHS-treated cows milked 1.5 times and 3 times the withholding times were sufficient even with the 95/95 tolerance limit.

Shorter excretion periods in cows milked more frequently as determined by TTSC method were confirmed by the analysis of variance (table 1). For all antibiotics the excretion times in cows milked 1.5 times versus 3 times were significantly different except for Omnygram®. On average also longer excretion periods of AMP and COL were observed in cows milked less frequently. Probably due to the large variation between excretion times in individual cows the differences were not significant for these two antibiotics.
From the different behaviour of antibiotic compounds in the two drugs containing more than one antibiotic it is confirmed, that not only the vehicle of the drug is important for excretion times in milk, but also the properties of the antibiotic itself.

Table 1: Withdrawal time (in hours) for the different antibiotic drugs in healthy cows (Least Square Means (LSQm) and standard error)

<table>
<thead>
<tr>
<th></th>
<th>Milking frequency per day¹</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3x</td>
<td>2x</td>
<td>1.5x</td>
</tr>
<tr>
<td>Cobactan® LC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CEF</td>
<td>71.8 ± 6.5 a</td>
<td>111.6 ± 4.8 b</td>
<td>99.9 ± 5.6 b</td>
</tr>
<tr>
<td>Procain-Penicillin G 3 Mio.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEN²</td>
<td>64.7 ± 4.6 a</td>
<td>65.7 ± 4.5 ab</td>
<td>96.3 ± 4.5 b</td>
</tr>
<tr>
<td>Nafpenzal® MC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEN³</td>
<td>51.1 ± 4.5 a</td>
<td>61.4 ± 4.7 ab</td>
<td>76.7 ± 5.3 b</td>
</tr>
<tr>
<td>NAF</td>
<td>31.8 ± 1.6 a</td>
<td>38.8 ± 1.7 b</td>
<td>48.0 ± 1.9 c</td>
</tr>
<tr>
<td>DHS</td>
<td>34.8 ± 3.9 a</td>
<td>48.9 ± 4.0 b</td>
<td>56.7 ± 4.6 b</td>
</tr>
<tr>
<td>Omnygram®</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td>82.9 ± 27.8 a</td>
<td>n.a.</td>
<td>123.5 ± 18.5 a</td>
</tr>
<tr>
<td>COL</td>
<td>62.3 ± 20.3 a</td>
<td>n.a.</td>
<td>69.5 ± 13.5 a</td>
</tr>
</tbody>
</table>

¹ Different letters 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

Other factors of significant influence on excretion time were days after calving for CEF (shorter excretion times in cows <100 d after calving) as well as number of lactation for PEN and DHS in Nafpenzal® LC experiments (shorter excretion times for cows in first lactation). The latter was no longer significant when body weight was included into the analysis.

3.2 Excretion of residues in cows with clinical mastitis
Only a limited number of excretion studies was possible in cows with clinical mastitis. The drug Cobactan® LC was selected for these studies, because in healthy cows large variability of excretion times as well as concentrations in milk exceeding the MRL close to the end of the withholding period were determined.

The excretion of CEF in milk in percent of the total amount applied varied between 4.4 % and 46.7 % for cows milked two times per day and between 11.1 % and 28.9 % for cows milked 1.5 times per day. The average excretion rate in milk was 26.7 % and 18.0 %, respectively and much lower than those observed in healthy cows (43.1 % for cows milked 2 times, 51.5 % for cows milked 1.5 times per day). Probably changes in permeability of the blood milk barrier or in pH of milk followed by increased absorption of antibiotics from the udder are the reasons for these differences. The extremely low excretion rate of 4.4 % was observed in one cow which had severe mastitis and fever. This may be explained by severe damage of the blood udder barrier thus allowing passage of a higher percentage of the antibiotic to blood serum.

The concentrations of CEF in milk after treatment are presented for individual cows milked 2 times per day in figure 5.
Large variation was found for excretion times in milk of individual cows. The time until concentrations fell below the MRL was between 38 and 72 hours. In none of the cows the MRL was exceeded at the end of the withholding time.

Also for cows milked 1.5 times per day large variation was observed. Times until concentrations in milk fell below the MRL varied between 48 and 64 h (data not shown).

Different excretion times were determined in individual cows treated repeatedly for different cases of mastitis with at least two weeks between different cases (table 2).

Table 2: Variation in excretion times of CEF in milk in individual cows with more than one case of clinical mastitis

<table>
<thead>
<tr>
<th>Cow</th>
<th>1st case (hours)</th>
<th>2nd case (hours)</th>
<th>3rd case (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1808</td>
<td>72</td>
<td>58</td>
<td>-</td>
</tr>
<tr>
<td>1868</td>
<td>48</td>
<td>48</td>
<td>-</td>
</tr>
<tr>
<td>1869</td>
<td>38</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>1878</td>
<td>62</td>
<td>72</td>
<td>-</td>
</tr>
<tr>
<td>1907</td>
<td>48</td>
<td>64</td>
<td>-</td>
</tr>
<tr>
<td>1915</td>
<td>48</td>
<td>48</td>
<td>-</td>
</tr>
</tbody>
</table>

1 milking frequency two times per day, except 1907 (1st case - 2 x, 2nd case 1.5 x per day) and 1868 (both cases 1.5 x per day), 2 cow received additional treatment with 650 mg CEF i.m.

The withdrawal times calculated by TTSC method and by analysis of variance are summarised for the two groups milked with different milking frequencies in table 3.
Table 3: Withdrawal times for CEF (hours) in cows with clinical mastitis in dependence on milking frequency per day

<table>
<thead>
<tr>
<th>Milking frequency per day</th>
<th>TTSC method Mean</th>
<th>95/95</th>
<th>Analysis of variance LSQM ± se²</th>
<th>sign.³</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 times (n=15)</td>
<td>54</td>
<td>66</td>
<td>57.7 ± 3.0 a</td>
<td></td>
</tr>
<tr>
<td>1.5 times (n=4)</td>
<td>52</td>
<td>108</td>
<td>45.5 ± 6.4 a</td>
<td></td>
</tr>
</tbody>
</table>

¹ 95/95 tolerance limit, ² LSQM=Least Square Mean, se=standard error, ³ different letters within column show significant differences (p<0.05)

The withdrawal times in cows with mastitis were much shorter than in healthy cows milked with the same milking frequencies after treatment with Cobactan® LC. One reason is probably the lower total amount of drug used for treatment of only one quarter compared to the worst case studies with treatment of 4 udder quarters in healthy cows. In addition, a lower percentage of the total amount of CEF was excreted via milk in cows with mastitis. Therefore it can be concluded that larger amounts of CEF were absorbed into blood serum and excreted via other ways. This was especially the case in one cow with fever where less than 5 % of the antibiotic were excreted via milk.

The means calculated by the TTSC method were very similar for the two groups milked with different intervals. When the 95/95 tolerance limits are included it has to be considered that for the group milked 1.5 times per day a large safety factor had to be included due to the low number of cows in this trial (n=4) versus 15 cows in the group milked 2 times per day. Between the two groups no significant differences of withdrawal times were determined by analysis of variance. The only factor of significant influence on excretion time was the average daily milk yield per cow, with shorter excretion times in high yielding cows (p<0.05). This factor was no longer significant when body weight was included into the analysis.

4. Conclusions

The excretion time of antibiotic residues in milk of healthy cows varied with different milking frequencies after intramammary treatment. For CEF, PEN, NAF and DHS significantly shorter excretion times were observed in cows milked 3 times per day compared to cows milked 1.5 times per day. Longest excretion times were observed for all antibiotics except for CEF in cows milked less frequently with 1.5 milkings per day.

Treatment trials in cows with clinical mastitis showed large variation in excretion times of individual cows and much shorter excretion times than in worst case studies in healthy cows. Although no significant differences were determined between groups milked 2 times or 1.5 times per day it should be considered that only one drug was tested in a low number of cows. A more pronounced influence of milking frequency may be observed for other drugs. Although results from studies in healthy cows can not necessarily be transferred to cows with clinical mastitis controlled milking of cows at least 2 times per day is recommended after treatment with antibiotics.

If cows in AM systems are milked in shorter intervals after treatment higher concentrations of residues in milk have to be expected. This could increase the risk for carry over of antibiotics into milk of the next cow milked at the same place, especially if failures in cleaning procedures occur.

According to the results presented prolonged excretion of residues in milk of individual cows in connection with milking frequencies deviating from regular milking times...
twice per day seem to be of minor importance for positive results of inhibitor tests in bulk tank milk when recommendations for treatment are followed.

5. References


Acknowledgements

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