Slov Vet Res 2014; 51 (2): 65 - 72
UDC 636.2.09:618.19-002:615.331:613.287.5:614.35

Original Scientific Article

INFLUENCE OF PROLONGED TREATMENT PROTOCOLS ON MAXIMUM RESIDUE LEVELS OF AMOXICILLIN CONCENTRATIONS IN BOVINE MILK

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Summary: The aim of this study was to evaluate two off-label protocols for the treatment of bovine mastitis with regard to antibiotic residues in milk and length of withdrawal periods. Forty-eight udder quarters infected with *Staphylococcus aureus*, from 19 dairy cows were included in the study. The animals were divided into two groups (A and B); Group A received intra-mammary and systemic intramuscular treatment with amoxicillin while Group B received intra-mammary treatment only. The impact of the treatment regime on antibiotic concentrations in milk was evaluated. All cows received six intra-mammary injections with cattle-labelled injectors in 12-h intervals, whereas cows in Group A were additionally treated intra-muscularly twice in 24-h intervals. Milk samples were taken in 12-h intervals up to 120 hours after the beginning of treatment, which was considered to be a cut-off value for the withdrawal period. Amoxicillin concentrations were measured using a Waters Xevo Triple Quad mass spectrometer (QQQ) and a Waters UPLC. Amoxicillin concentrations were significantly higher in Group A than in Group B in all measured intervals. Antibiotic concentrations significantly decreased at 72 h (Group B) and 84 h (Group A) after the beginning of treatment. At the end of the producer-suggested withdrawal period, concentrations of amoxicillin were above MRL in 65% of udder quarters in Group B and in 40% of udder quarters in Group A.

Key words: amoxicillin; milk; *Staphylococcus aureus*; mass spectrometry

Introduction

Mastitis is one of the most significant and costly diseases in the dairy industry (1, 2). Over 100 different microorganisms can cause inflammation of the mammary gland; in the majority of cases, staphylococci, streptococci and coliform bacteria are involved. Mastitis pathogens are usually categorized as contagious, such as *Streptococcus agalactiae* and *Staphylococcus aureus,* environmental, such as *Escherichia coli*, or skin flora opportunists, such as coagulase-negative species of staphylococci (3).

Received: 18 March 2013
Accepted for publication: 8 April 2014

Staphylococcus aureus (S.aureus) is contagious mastitis pathogen and one of the most prevalent major mastitis pathogens in the United States and Europe (4).

In dairy herds, *S. aureus* mastitis can be usually successfully controlled and even eradicated using adequate preventive measures; however, these procedures are time consuming and costly (5). Therefore, in recent years more emphasis has been placed on the treatment of infected udder quarters. Despite that, cure rates concerning the treatment of clinical and especially subclinical *S. aureus* mastitis are still disappointing, and the selection of animals for therapy is crucial in order to achieve reasonable results (6). Reported cure rates for *Staph. aureus* mastitis range considerably; for example, cure

rates for subclinical *Staph. aureus* mastitis range from 4 to 92% (7, 8, 9). The probability of cure depends on the cow, the pathogen and treatment factors. Cure rates decrease with the increasing age of the cow, increasing somatic cell counts, the increasing duration of infection, increasing bacterial colony counts in milk before treatment, and the increasing number of quarters infected (4). Antibiotic preparations for mastitis treatment introduced in the previous 25 years have not been a significant improvement over products that had been in use for a longer period. Moreover, in recent years, no new antibiotics have been released on the market. In order to improve cure rates, treatment regimens have been extended, and a combination of intra-mammary and systemic treatment has been employed (10).

It is well documented that extended therapy regimes improve the bacteriological cure rates of *S. aureus* mastitis (10, 11, 12). Due to the course of the inflammation process in the infected mammary gland and the formation of scar tissue and micro-abscesses (13, 14), the common 1- to 2-day intra-mammary therapy may, in the majority of cases, be too short. Therefore, at least one initial systemic injection should be administered in combination with a 3-day intra-mammary treatment. Benefits of extended treatment protocols, such as higher proportions of cure, resulting in decreasing somatic cell counts, reduced risk of transmission and improved marketability of milk, must be weighed against several drawbacks, including the price of the antibiotic, loss of milk due to withdrawal, increased risk for residues in the milk, and the potential of infecting the mammary gland through repeated infusions via the teat canal (4, 15, 16). Moreover, it is also emphasized that antibiotic treatment will not control new mammary gland infections with *S. aureus*; therefore, only adequate preventive measures will provide lasting results.

Additionally, an extension of the therapy regime prescribed by the producer means that the withdrawal periods must also be prolonged. Within the European Union, veterinarians are allowed to prescribe drugs in an off-label manner, regarding animal species, route of administration or dosage, but are then obligated to ensure that residues do not enter the food chain. Residues are of concern due to possible allergic reactions in people, potential buildup of antibiotic-resistant organisms in people, and the inhibition of starter cultures used in production of cultured milk products, such as yogurt and cheeses (17). According to the provisions in the directive of the European Parliament, 2001/82/EC, in case of off-label treatment the withdrawal period for milk "shall not be less than 7 days" (18).

The objective of the present study was to evaluate the impact of two different extended off-label treatment protocols (one using a combination of intra-mammary and systemic applications and the other intra-mammary applications only) on antibiotic concentrations in milk and the length of the withdrawal period. This study also addresses the validity of the EU directive requiring a 7-day withdrawal period.

Materials and methods

Selection of animals

Nineteen lactating dairy cows (Holstein Friesian) and 48 udder quarters infected with *S. aureus* were included in the study between January and March 2011. The animals were in different phases of lactation and had no perceptible signs of inflammation of the mammary gland; subclinical *S. aureus* mastitis was established during routine milk sampling. None of the cows received any antibiotic preparations two months prior the start of the study. Standard lactation (SL), days in milk (DIM), daily milk yield (DY), body condition score (BCS), and pregnancy status were calculated from farm data records on a cow level (Tables 1 and 2). The study was conducted on a base of routine milk sampling, diagnostics and treatments in accordance to the Slovenian Veterinary Ethical Code. None of the animals was additionally exposed to any unapproved matter.

In Group A, 23 quarters were treated belonging to 9 cows, and in Group B 25 quarters belonging to 10 cows. On average, 2.6 ± 1.0 and 2.5 ± 0.8 quarters per cow were treated in Groups A and B, respectively. In both groups, the number of treated quarters varied from one to four (Table 1, Table 2).

Milk sampling for bacteriological determination

In a yearly routine, i.e. milk sampling for the microbiological status of the herd, milk samples were taken from individual udder quarters of

cows and collected into autoclaved glass tubes following teat disinfection with 70% alcohol, after discarding first three streams of milk. Collected samples were kept cool and sent to a lab the same day for bacteriological determination.

Milk samples were later streaked with a sterile swab within 24 h on quarter plates of washed blood agar with 5% sheep blood and incubated at 37°C. After 48 h, the plates were examined for aerobic bacterial growth. Gram-positive cocci were considered to be *Staphylococcus* or *Micrococcus* species if they were catalase positive. Differences in hemolysin production were classified visually by an experienced observer as either β-hemolysin positive or negative. The slide coagulase test was performed as described by the manufacturer of the rabbit plasma (Biokar diagnostics, France). Later, the API-Staph test (Biomerieux, Macy I´Etolle, France) was used for the final determination of *S. aureus*.

Treatment protocol

Cattle-labeled products for intramuscular (i.m.) and intra-mammary (i.mm) applications containing amoxicillin and clavulanic acid were used. Animals were randomly divided in two groups (Table 1 and 2) and treated by two off-label protocols. Cows in Group A were treated with a combination of systemic and intra-mammary treatment, twice in 24-h intervals with 7 mg amoxicillin-trihydrate and 1.75 mg of clavulanic acid in the form of potassium clavulanate per kilo body weight intramuscularly (Synulox® RTU, Pfizer Luxembourg SARL, Luxembourg), in addition to six intra-mammary injections (Synulox® LC, Pfizer Luxembourg SARL, Luxembourg) of 200 mg amoxicillin-trihydrate, 50 mg of clavulanic acid in the form of potassium clavulanate and 10 mg prednisolone in 12-h intervals. Cows in Group B were treated exclusively with intra-mammary (i.mm.) injections of 200g amoxicillin-trihydrate, 50 mg of clavulanic acid in the form of potassium clavulanate and 10 mg prednisolone six times in 12-h intervals (Table 3).

Milk sampling

To establish the excretion rate of the tested antibiotic product, milk samples of foremilk from treated udder quarters were collected in 12-hour intervals from zero to 120 hours after the start of treatment, which was considered as a cut-off value for withdrawal period. Additional samples were taken 240 h post treatment. Samples were kept deeply frozen at -20°C until analysis.

Sample preparation

1 mL of milk precipitated with acetonitrile (ACN) was mixed in vortex, followed by 10-min centrifugation step at 4000 RPM. The supernatant was filtrated through a 0.45 µl filter. The 10 µl filtrate were injected onto the LC/MS/MS.

LC/MS/MS instrumentation

LC conditions for chromatographic separation were as follows: column, HP ZORBAX Eclipse plus C18 column with dimensions 2.1 x 100 mm i.d., dp 1.8 µm; column temperature at 30°C; mobile phase, (A) 5% ACN and 95% H_20 with 0.1% HCOOH and (B) ACN with 0.1% HCOOH; flow rate, 0.5 mL min^{-1}. Gradient elution was performed (initial gradient: 100% A, 0.5 min: 100% A, 0.51 min: 50% A, 1.7 min: 50% A, 1.71 min: 100% A, 2.2 min: 100% A).

MS conditions

The Waters Xevo Triple Quadropole mass spectrometer was used in the ESI+ mode. The following tune parameters were used: capillary, 2.5 kV; cone, 20 V; source temperature, 150°C; desolvation temperature, 500°C. The instrument was operated in the multiple reaction mode, using the following transitions: m/z = 366.2>113.9 (quantification trace) at collision energy 18 eV, m/z = 366.2>208 (confirmation trace) at collision energy 12 eV. Amoxicilin retention time is 0.58 min. Recovery on MRL value is 85%; the relative standard deviation is 25% on the same concentration level, and the correlation coefficient is 0.89.

Statistical evaluation of results

Data concerning standard lactation (SL), days in milk (DIM), daily milk yield (DY) and body condition score (BCS) were first tested for normality of distribution using a Kolmogorov-Smirnov test. In case that data were normally

Table 1: Animal data of lactation number, standard lactation (305 days), day in milk, number of infected quarters, body condition score and pregnancy status. Group A

Cow No	Lactation No	SL (L)	DIM (L)	DY	No of quarters infected	BCS	Pregnancy status
1	2	9809	358	22	2	3.50	+
2	2	8144	396	15	1	4.00	+
3	1	9153	150	30	3	3.00	+
4	1	8132	186	26	4	3.50	+
5	3	5893	137	26	2	3.75	-
6	1	8210	213	22	3	3.25	+
7	2	8266	183	30	4	3.25	+
8	6	9105	135	29	2	3.00	-
9	2	9231	143	31	2	2.75	-
Mean	2.2	8438.1	211.2	25.7	2.6	3.33	
SD	1.6	1129.3	98.0	5.2	1.0	0.40	
Median	2.0	8266.0	183.0	26,0	2.0	3.25	

Table 2: Animal data of lactation number, standard lactation (305 days), day in milk, number of infected quarters, body condition score and pregnancy status. Group B

Cow No	Lactation No	SL (L)	DIM (L)	DY (l)	No of quarters infected	BCS	Pregnancy status
1	7	6782	211	12	3	3.75	+
2	5	6115	153	21	3	3.50	+
3	3	8491	294	28	2	3.75	+
4	3	9523	334	18	1	3.75	+
5	4	9293	127	33	3	3.25	-
6	4	6824	113	24	4	3.50	+
7	1	9178	122	34	2	2.75	-
8	4	9321	116	40	3	2.75	-
9	3	7322	185	24	2	3.75	+
10	3	10657	99	52	2	2.50	-
Mean	3.7	8350.6	175.4	28.6	2.5	3.33	
SD	1.6	1493.5	81.3	11.6	0.8	0.49	
Median	3.5	8834.5	140.0	26.0	2.5	3.50	

Table 3: A timeline diagram of drug administration in infected quarters in Groups A and B

	0 hour	12 hours	24 hours	36 hours	48 hours	60 hours
Group A	i.mm. i.m.	i.mm.	i.mm. i.m	i.mm.	i.mm.	i.mm.
Group B	i.mm.	i.mm.	i.mm.	i.mm.	i.mm.	i.mm.

distributed, a parametrical t-test test was used to test the differences between Groups A and B, whereas a Mann-Whitney Rank Sum Test was used if data were not normally distributed.

Regarding amoxicillin concentrations, the Kolmogorov-Smirnov test showed that data were not normally distributed; therefore, a $\log_{10}$-transformation of the amoxicillin concentrations was performed to normalize the data. Antibiotic concentrations ($\log_{10}$) at different time samplings in each group were tested using a One-Way Repeated Measures Analysis of Variance. In case of a significant difference among sampling, a pairwise comparison was performed with the Holm-Sidak method using Bonferroni correction. Amoxicillin concentrations ($\log_{10}$) between Groups A and B at each time sampling was compared using t-test.

Values of $P<0.05$ were considered significant for all analyses. SigmaStat 3.5 (SYSTAT Software Inc.) software was used for the statistical evaluation of the results.

Results

Cows in Groups A and B did not differ according to standard lactation, days in milk, daily milk yield and body condition score ($P>0.05$).

Figure 1 shows $\log_{10}$ of amoxicillin concentration in milk in quarters of animals in Groups A and B. Concentrations of amoxicillin in milk differ between Groups A and B from 12 to 84 h after the first treatment ($P<0.05$). Antibiotic concentration significantly decreased 48 and 72 h after the beginning of treatment in Groups A and B, respectively (Figure 1). From data of Table 4, it is evident that after 120 h from the beginning of treatment 65.2% of quarters in Group A and 40.0% in Group B show the concentration of amoxicillin above MRL (MRL = 4µg(kg); $\log_{10}$MRL = 0.6). According to cow level, 66.7 and 20.0% cows in Groups A and B, respectively, show concentration of amoxicillin above MRL. After 240 h, in three quarters from Group A (2 cows), amoxicillin concentration was above zero, but below MRL, whereas no detectable amoxicillin concentration was observed in any of assayed quarters from Group B (Table 4, Figure 1).

Discussion

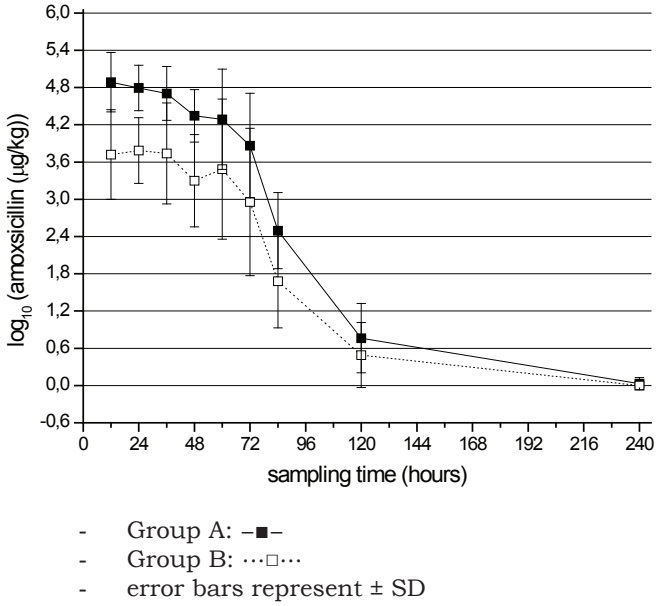
The aim of this study was to evaluate the influence of two mastitis treatment protocols on the presence of amoxicillin residues in bovine milk. Amoxicillin and clavulanic acid are considered to be the treatment of choice in case of penicillin-resistant *S. aureus* strains (4, 19). The treatment protocols tested in the present study are extended protocols based on the results of several studies that have shown that prolonged regimes are the most efficient and cost-effective in the treatment of *S. aureus* mastitis (9, 10, 11). Gillespie *et al.* (20) also found considerably higher cure rates in five- to eight-day therapies compared to two-day therapy. Sol *et al.* (12) observed 2.3-times higher rates of bacteriological cure using extended treatment protocols compared to standard treatments. In contrast, cattle-label antimicrobials for intra-mammary treatment are usually registered for between one and three administrations in 12- to 24-hour intervals. This may not allow drug concentrations at the site of action to exceed the minimal inhibitory concentration for a sufficient period, which potentially influences cure rates, as drug distribution to the site of infection is especially important in the case of *S. aureus* with which micro-abscess and intracellular habitation are common.

According to the manufacturer, the withdrawal periods for one intramuscular and one intra-mammary administration of amoxicillin used in our study were 24 h and 72 h, respectively. Amoxicillin concentrations were significantly higher in Group A (combined intra-mammary and systemic treatment, Figure 1) than in Group B (intra-mammary treatment only, Figure 1) in all measured intervals, except at 120 h and 240 h, where concentrations did not differ significantly between groups. This means that irrespective of treatment duration, higher antibiotic concentrations in milk were achieved by combining intra-mammary and systemic applications. With this protocol, amoxicillin concentrations progressively decreased but remained high until 72 h after the beginning of treatment. Amoxicillin concentrations in Group B, however, differed between sampling intervals and showed overall lower levels. Concentration of amoxicillin decreased significantly after 72 h in Group A and after 84 h in Group B. The sampling interval at 120 h was calculated as the end of the withdrawal period according to drug manufacturer (72 h after last intra-mammary administration). The sampling interval at 240 h followed withdrawal periods defined in the directive of the European

Table 4: Comparison of amoxicillin concentrations in milk quarters of animals at all sampling times according to treatment in Groups A and B

Amoxicillin concentration (μg kg^{-1})	Sampling time (h)								
	12	24	36	48	60	72	84	120	240
Group A (cows N= 9; quarter N=23)									
Mean	126608.9	93358.2	85561.2	35849.3	47930.6	23952.3	750.9	13.2	0.3
SD	130062.4	100986.7	80734.4	33481.2	51602.7	25021.0	990.6	16.5	0.9
Median	76123.0	50936.0	63490.0	21648.5	33684.0	13926.0	403.0	9.0	0.0
Min	9397.0	11863.0	6767.0	3725.0	79.0	190.0	6.1	0.0	0.0
Max	529042.0	414367.0	304143.0	135189.0	198146.0	68597.0	3724.0	70.0	3.2
Quarters above MRL* (%)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	65.2	0.0
Cows above MRL* (%)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	66.7	0.0
Group B (cows N= 10; quarters N=25)									
Mean	16064.2	12973.0	17621.1	7081.3	21056.5	7150.0	143.3	5.8	0.0
SD	23835.3	20318.6	24654.7	13810.3	33788.9	12599.3	222.4	8.0	0.0
Median	4295.0	5408.5	6924.0	1841.0	4135.0	1541.0	72.0	2.4	0.0
Min	177.0	475.0	144.0	55.0	35.7	4.8	0.0	0.0	0.0
Max	79182.0	84699.0	94773.0	60358.0	150014.0	45915.0	854.0	28.0	0.0
Quarters above MRL* (%)	100.0	100.0	100.0	100.0	100.0	100.0	92.0	40.0	0.0
Cows above MRL* (%)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	20.0	0.0

*: MRL = 4 μg/kg



- Group A: –■–
- Group B: ···□···
- error bars represent ± SD

Figure 1: Amoxicillin concentration (log10) in milk in quarters of animals treated in Group A and B

Parliament, 2001/82/EC (18), regarding off-label use of a veterinary medical product (168 h after last intra-mammary administration). At 120 h, amoxicillin concentration was above the MRL in 65.2% of quarters in Group A and in 40.0% in Group B (Table 4). At 240 h, MRL was exceeded in none of the analyzed samples. Considering the results of Stockler *et al.* (21), amoxicillin concentrations measured in the foremilk samples in our study could be higher than concentrations potentially measured in a quarter's bulk milk. We are also aware that concentrations measured at the end of the withdrawal period could be lower on a cow level due to the dilution effect. Regardless of the treatment protocols and levels of antibiotic concentrations, our results also indicate that the duration of therapy significantly influences withdrawal periods. In our previous study (22), the clearance of water-soluble benzylpenicillin procaine from milk was much faster compared to the oil-soluble preparation used in this study; therefore, the withdrawal period may depend not only on the type of beta-lactam, but also on the

supplements, such as mineral oils and Ca-Na-Aluminosilicate in injectors registered for intramammary use.

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VPLIV PODALJŠANIH TERAPEVTSKIH PROTOKOLOV NA OSTANKE AMOKSICILINA V KRAVJEM MLEKU

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Povzetek: Namen študije je bil ovrednotiti vpliv dveh terapevtskih protokolov z izredno uporabo zdravil pri vnetju mlečne žleze na pojav ostankov zdravil v mleku in dolžino karence. V raziskavo je bilo vključenih oseminštirideset mlečnih četrti, okuženih z bakterijo *Staphylococcus aureus*, 19 krav molznic. Živali so bile razdeljene v dve skupini (A in B), skupina A je bila zdravljena intramamarno in intramuskularno, skupina B pa le intramamarno. Ovrednoten je bil vpliv terapevtskega protokola na koncentracije antibiotika v mleku. Vse krave so bile zdravljene s 6 injektorji za intramamarno uporabo tri dni na 12 ur, medtem ko so bile krave v skupini A dodatno intramuskularno zdravljene še z enakim antibiotikom, registriranim za parenteralno uporabo dvakrat v razmaku 24 ur. Mlečni vzorci so bili odvzeti v 12-urnih intervalih do 120 ur po zdravljenju, ki je predstavljalo točko preloma za določitev dolžine karence. Koncentracije amoksicilina so bile zmerjene na masnem spektrometru Waters Xevo Triple Quad (QQQ) in Waters UPLC. Koncentracije amoksicilina v mleku so bile značilno višje pri skupini A v primerjavi s skupino B v vseh meritvenih intervalih. Koncentracije antibiotika so se pri skupini B značilno znižale 72 ur, pri skupini A pa 84 ur po začetku zdravljenja. Ob izteku karence, določene pri proizvajalcu, je bilo v skupini A 65 % vimenskih četrti nad dovoljeim MRL, pri skupini B pa 40 %.

Klučne besede: koncentracije amoksicilina; kravje mleko; *Staphylococcus aureus*; masna spektrometrija