Evaluation of a standard protocol for drying off and drying off therapy in dairy cows based on the comparison of two different commercial antimicrobials

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Running title:

Drying off therapy in a dairy farm

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Abstract

This study aims to evaluate two commercial antibiotics for dry-off. Ninety-five Friesian cows and 380 quarters were included. Cows were classified as Control and Subclinical Mastitis based on somatic cell count. The California Mastitis Test and the teat end score have been performed. Quarters were randomly treated with Cloxacillin and Ampicillin (TA) and Cephalexin (TB). The effect of the therapy (TA vs TB) was estimated by X2 analysis based on the Wald test. The preservative and therapeutic action of TA vs TB were evaluated by the Kruskall-Wallis test. TB showed a statistically significant therapeutic effect in the control group, that might be related to the pharmacological activity of the two antibiotics. Also, the subclinical mastitis group most commonly presented more quarters affected compared to the control group, leading to a worse improvement despite proper therapy. In conclusion, an abruptly dried off, the California mastitis test, teat end score and somatic cell count evaluation, as long as microbial herd data might represent key concepts for an efficient drying off standard protocol in a dairy farm. In line with the herd bacterial population, both TA and TB might be employed for drying off therapy.

Key words: Veterinary, dairy cow, mastitis, dry period, drying-off therapy.

Introduction

The dry period is defined as the nonlactating period prior to parturition in dairy cows. Usually, this period begins at the end of the lactation curve, whom shape, and length is strongly influenced by several environmental and genetic factors (Macciotta et al. 2011). The length of the dry period is about 60 days in Europe and United States of

America (Capuco et al. 1997; Annen et al. 2004). The dry period has a critical role for udder health, due to an increased risk of intra-mammary infections (IMI) during this time (Bradley and Green 2000; Whist and Østeras 2007). Despite a good dry period management, some animals appear more prone to new IMI than others and may show clinical mastitis in the next lactation (Hogan and Smith 2003). The most frequently isolated microorganisms at dry-off are *Streptococcus* spp., coagulase-negative staphylococci (CNS), *Staphylococcus aureus* and *Corynebacterium* spp. (Pantoja et al. 2009). Dry cow therapy (DCT) is an intra-mammary treatment with an antibiotic, administered at the beginning of the dry period. Since many years, the treatment of IMI at drying off has been a basis for mastitis control and management (Bradley and Green 2000). Dry cow therapy eliminates existing IMI and preventing new IMIs (Bradley and Green 2000; Dingwell et al. 2004; Whist and Østeras 2007). The elimination of infection and the prevention of new IMI in the dry period is easier than during lactation. The drug is not milked out, so the antibiotic can remain longer in the udder. Moreover, the absence of regular milking reduces the exposition to pathogens by teat penetration (Berry et al. 2004; Kashif et al. 2016).

The most common drugs used as dry-off therapy are intra-mammary tubes containing antibiotics such as penicillin, cloxacillin, cephalosporin and spiramycin (Kashif et al. 2016). Despite the large amount of literature about dry-off therapy strategies, usually the decision concerning which antibiotic therapy would be better in a specific herd is still made by drug popularity and farmer’s preference and not based on scientific evidences.

The aims of the present study were to evaluate the effects of two different commercial antibiotics at dry-off in the same herd on the mammary microbiological populations, in order to set a standard protocol for the dry off therapy in line with isolated bacteria.

Materials and methods

Animals

The present study was carried out in an intensive dairy farm located in the North of Italy. A total of 108 Italian Holstein Friesian cows in the same management conditions were considered for the trial. An owner's written consent for participating on the study have been recorded. All the experimental procedure were in compliance with the 2010/63/UE directive about the protection of animals in the scientific experiments. Cows were between 210 and 220 days of gestation and were considered healthy based on the complete physical examination. Animals included in the trial were not subjected to antibiotic and/or nonsteroidal anti-inflammatory drugs (NSAIDs) treatments for 30 days period before the admission time and did not showed udder or milk abnormalities at dry-off. All the included animals were observed from drying-off until 15 days of lactation. The drying off standard protocol proposed in this study has been carried out as follow. All eligible cows were dried off abruptly. During the last milking session, the teat end score (TESa) was recorded for each quarter as reported in literature (Hamann and Mein 1996; Neijenhuis et al. 2000), along with the udder evaluation. The California Mastitis Test (CMTa) was used as the screening test for clinical or subclinical mastitis detection (Barnum and Newbould 1961). A sample has been considered positive for CMT ≥ 1 (Blowey and Edmondson 2010). Before the antibiotic administration, a sterile milk sample for bacteriologic analysis was collected from positive CMT quarter in a 15 mL tubes (Falcon®, BD, Italy) and was stored at 4°C ± 2°C until the time of analysis.

All the samples were processed within 3 hours. After these evaluations, the animals were treated with Cloxacillin and Ampicillin (Cloxalene Max, Fatro, Italy), or Benzathine Cephalexin (Rilexine, 500 HL, Virbac, Italy) as drying-off therapy, despite the CMT results. Dry-off cows were then transferred to a separate box, located in a different barn. The dry-cow's box was composed by a pen for the daily exercise, in between the feeding area, where all animals were fed at the same time. Moreover, in this area there was a resting zone composed by an appropriate number of cubicles bedded with daily-changed straw. At dry-off, cows were fed a low energy density diet, administered as total mixed ration (TMR) two times per day (Table 1). At the inclusion time, animals were divided in two groups (Treatment A and Treatment B) based on the drugs supply at the farms. Treatment A (TA) quarters received 600 mg of Cloxacillin and 300 mg of Ampicillin (Cloxalene Max, Fatro, Italy), while treatment B (TB) quarters received 375 mg of Benzathine Cephalexin (Rilexine, 500 HL, Virbac, Italy). The whole 4 quarters of a single cow were treated with the same antibiotic. For a better classification of the cows, the average somatic cells count (SCCa) of the 4 quarters has been evaluated at the inclusion time by the MilkoScan (FOSS, Italy). Thus, animals were retrospectively classified as "C cows" when the SCC was < 150'000 cells/mL for primiparous and < 250'000 cells/mL for multiparous cows and as "SCM cows" when the SCC was > 150'000 cells/mL for primiparous and > 250'000 cells/mL for multiparous (de Haas et al. 2008; Windig et al. 2010). The "C cows" has been considered as control animals. A second evaluation took place 15 days after parturition for each quarter included. The same parameters collected at the inclusion time were considered: 1) teat end score for each quarter (TESb); 2) CMT performed for each quarter 15 days after parturition (CMTb); 3) the average SCC (SCCb) of each single quarter.

Bacteriology analysis

To isolate the mastitis agents and to screening the microbials population of the studied herd, 0.01 mL of each CMTa positive milk sample was streaked on blood agar plates containing 10% sheep blood. Each plate was incubated aerobically at 37 °C for 24-48 h. After observation of colony morphology and hemolytic patterns on blood agar, isolates were submitted to Gram staining, catalase and oxidase testing and additional biochemical and metabolic evaluations as needed.

Gram-negative organisms were successively identified by sowing on appropriate selective and differential media; furthermore, enzyme activities, acid production from different carbohydrates, assimilation of various substrates were determined using commercial systems- API ZYM, API 20E and API 20NE (BioMerieux ®) according to the manufacturer’s instructions.

Staphylococcus spp., grown on Baird Parker medium with the typical halo associated with lecithinase positivity and characterized by typical zones of complete and incomplete hemolysis and nonhemolytic Staphylococcus spp. that had a positive tube test for free coagulase were classified as Staphylococcus aureus; all other staphylococci were classified as CNS. Moreover, all isolates belonging to Staphylococcus genus were also identified using API Staph (BioMerieux ®). The other Gram-positive cocci, grown on blood agar plates and negative for catalase test, were phenotypically identified by means of API 20 Strep (BioMerieux ®).

Statistical analysis

Data concerning SCCa and SCCb, CMTa and CMTb, type of treatment (TA vs TB) and TESa and TESb of C and SCM groups were expressed as prevalence.

Data obtained in this work were analyzed by R software (R Development Core Team). Firstly, the effect of the therapy (use of the antibiotics TA or TB) on the number of positive or negative teats to CMTa were estimated by $\chi 2$ analysis based on the Wald test. The analysis was repeated both for cows classified as “C” or “SCM”.

Subsequently, we estimated the different effect of the two antibiotics by the non-parametric Kruskall-Wallis test. This analysis was repeated in two different situations: firstly, we evaluated the protective action of the two antibiotics considering the percentage of negative CMT teats that remain unchanged after the treatment; while the therapeutic effect was estimated considering the percentage of positive CMT teats that became negative after treatment.

Results

A total of 380 quarter in 95 cows were included, from 46 primiparous cows (48%) and 49 multiparous cows (52%). Thirteen animals from the 108 enrolled were excluded from the study for the presence of only three functional udder quarters or because of treatments during the 30 days before the inclusion time. Cows were in first to fifth lactation, with an average of 4.5 (3-7) years old, average body weight of 660 kg (520-710) and average body condition score (BCS) of 3.25 (2.5-3.75).

One hundred and thirty-six/380 quarters (referred to 34 cows) received TA while 244/380 quarters (referred to 61 cows) were treated with TB. Based on SCCa evaluation, a total of 29 cows (18 primiparous and 11 multiparous) were included in the C group, while 66 cows (28 primiparous and 38 multiparous) were included in the SCM group. A total of 32 quarters belong to C group were treated with TA, while 84 quarters were treated with TB. A total of 104 quarters belong to SCM group were treated with TA, while 160 quarters were treated with TB. Data concerning SCCa, CMTa and TESa of C and SCM groups were reported in Table 2.

A total of 68 out of 380 milk samples were CMTa positive, thus they were sampled for bacteriologic analysis. Fiftythree out of 68 milk samples (78%) were culture negative, 9 (13%) were positive for environmental pathogens (CNS and Serratia), while the remaining 6 samples (9%) were positive for contagious pathogens. The pH was out of the normal range (6.5-6.7) in 41 out of 68 (60%) milk samples (Ruegg e Erskine, 2015). The average pH value was 6.8±0.2.

Results concerning CMTb and TESb of C and SCM cows, grouped based on SCCa are reported in Table 3, while results concerning CMTb and TESb of C and SCM cows, grouped based on SCCb are reported in Table 4.

Results concerning changing between CMTa vs CMTb in TA vs TB cows, classified based on SCCa are reported in Table 5.

Table 6 showed the result of contingency analysis related to the number of quarters treated with TA and TB. Statistical differences between expected and observed number of quarters were revealed for SCM cows ($p < 0,001$) with higher number of positive quarters observed for TA.

Results concerning the effect of the different antibiotic on teat in a preventive (expressed as % of teats that continued to show CMT negative after treatment) and therapy (expressed as % of teats that showed CMT negative after treatment) are reported in Table 7. Different effect between the two treatments was only observed only for the C cows ($p = 0,022$) during therapeutic action, while no difference was revealed for SCM cows (p = 0,990). On the contrary, the preventive effect was similar between TA and TB.

Discussion

Dry period is crucial for two different reasons: 1) high rates of IMIs healing during this period and 2) the develop rates of new IMIs during this period are higher than during the lactating period (Dingwell et al. 2003; Halasa et al. 2009). For these reasons, intramammary antibiotics are used to treat any existing IMI at dry-off and to prevent new IMI during the dry period. However, the choice of the best antibiotics therapy would be set according to the kind of mammary pathogens. Prudent use of antibiotics is recommended to prevent the development of antimicrobial resistance. The isolation of different pathogens and their sensitivity or resistance to some antibiotics is the key to choose the most suitable antibiotics to avoid the antimicrobial resistance (Scherpenzeel et al. 2014). The whole population of this study presented no udder or milk abnormalities at the dry-off but only CMT alterations, with a prevalence for subclinical mastitis of 18%, which is slightly lower value compared with data reported in literature (Busato et al. 2000; Gianneechini et al. 2002). However, our study only evaluated cows at drying-off, while data presented in literature usually came from the screening of the whole herd. This might explain the differences in results. Reported prevalence of infection at dry-off, due to any pathogen, ranges from 28 % to 50 % at cow level (Rindsig et al. 1978; Browning et al. 1994). Our results are slightly lower compared with other authors. However, the incidence of infections at dry-off in a herd could be influenced by many factors and this might explain the differences found in prevalence (Torres et al. 2008).

Otherwise, the high prevalence of subclinical mastitis obtained in this study, confirmed that subclinical mastitis still represents an important problem in the dairy cow industry (Pisoni 2007). Thus, screening for subclinical mastitis at the time of dry-off is mandatory for keeping a high standard of udder and milk health and hygiene.

The screening method choose in the present study was the CMT because it still represents the commonest used one in the field (Ruegg e Erskine 2015). Also, literature recommended the use of CMT for identification of IMI when herd prevalence of IMI is lower than 15% (Torres et al. 2008). Authors knew the average prevalence of IMI from the history of the herd. However, samples CMT positive were tested for BE and the prevalence of positive BE was 22%. Despite CMT is largely used in dairy practice, our results confirmed the too high sensibility of the test (Bradley et al. 2012; Zecconi and Zanirato 2013; Sgorbini et al. 2014). The CMT may be influenced by several factors, i.e. the time of sampling (morning vs evening) or the season (summer vs fall) (Bradley et al. 2012; Zecconi and Zanirato 2013), the storage and processing of the sample (Viguier et al. 2009; Bradley et al. 2012), udder inflammation other than infectious problems (i.e. trauma, alimentary management, milking procedures) (Zecconi and Zanirato 2013). Thus, for a more proper use of antibiotic therapy at dry-off, the BE might be considered the gold standard test for drive the decision (Ruegg and Erskine 2015). *Staphylococcus* spp. was the most common bacterium isolated in our population, as confirmed by literature (Torres et al. 2008; Zecconi and Zanirato 2013; Ruegg and Erskine 2015). Compared with TA, TB showed a statistically significant therapeutic effect in “C cows” while no difference where observed for “SCM cows”. Cloxacillin (TA) represents one of the most widely used antibiotics for the drying off in dairy cows (Halasa et al. 2009; Bhutto et al. 2011). However, Cephalexin (TB) has been successfully used in the treatment of subclinical mastitis of lactating cows (Tiwari et al. 2000). The difference in therapeutic effect found in the present study might be related to the pharmacological activity of the two antibiotics. Also, “SMC cows” most commonly had affected more quarters than “C cows”, leading to a worse improvement despite a proper therapy. Further studies with an increasing number of animals included might complete the present findings.

Conclusions

In conclusion, an abruptly dried off, the CMT, TEC and SCC evaluation, as well as microbiological examination might represent key concepts for an efficient drying off standard protocol in a dairy farm. The SCC evaluation is essential to find subclinical mastitis and to decide to treat the single quarter of the udder. The pathogens isolations before the drying off is the key to choose the ideal antimicrobial to treat it. In line with the herd bacterial population, both TA and TB might be employed for drying off therapy.

Acknowledgement and/or disclaimers, if any

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Table 1. Dry matter concentrations and chemical composition of the close-up dry (CUD) diets fed to Holstein Friesian cows.

CUD Diet	
Ingredients	Percentage
Corn silage	45.46
Alfalfa hay	5.00
Corn grains	18.28
Cottonseed hulls	11.17
Soybean meal	8.38
Cottonseed with lint	5.2
Prolak	2.55
Springer minerals	2.92
Salt	0.5
Sodium bicarbonate	0.54
Chemical composition	Percentage (DM basis)
DM (%)	62.07
CP	14.88
Sol CP[1]	29.25
ADF	26.17
NDF	40.53
EE[2]	3.81
TDN	67.22
NE_L (Mcal/Kg)	1.52
K^+	1.14
Na^+	0.40
Cl^-	0.82
S^{-2}	0.33
Ca^{+2}	1.07
P^+	0.38
Calculated DCAD[3]	+19.4

[1]Percentage of the CP

[2]Ether extract

[3]Meq/100g DM

Table 2. Data concerning the evaluation of SCCa, CMTa and TESa scores performed in C and SCM groups. Legend: SCCa – somatic cells count at inclusion time (dry-off); CMTa – california mastitis test at inclusion time (dry-off); TESa - teat score for each quarter; TA – treatment A (600 mg of Cloxacillin and 300 mg of Ampicillin); TB – treatment B (375mg of Benzathine Cephalexin); C group – control group; SCM group – subclinical mastitis group.

SCCa (cell/ml)	CMTa	TESa score 1		TESa score 2		TESa score 3		TESa score 4	
		TA	TB	TA	TB	TA	TB	TA	TB
C group	Positive	1/32	0/84	2/32	4/84	0/32	2/84	0/32	0/84
	Negative	26/32	68/84	3/32	10/84	0/32	0/84	0/32	0/84
SCM group	Positive	11/104	4/160	17/104	14/160	2/104	8/160	0/104	3/160
	Negative	54/104	94/160	19/104	32/160	1/104	3/160	0/104	2/160

Table 3. Data concerning the evaluation of CMTb and TESb scores performed in C and SCM grouped based on SCCa. Legend: SCCa – somatic cells count at inclusion time (dry-off); CMTb – california mastitis test 15 days after parturition; TA – treatment A (600 mg of Cloxacillin and 300 mg of Ampicillin); TB – treatment B (375mg of Benzathine Cephalexin); C group – control group; SCM group – subclinical mastitis group.

SCCa (cell/ml)	CMTb	TESb score 1		TESb score 2		TESb score 3		TESb score 4	
		TA	TB	TA	TB	TA	TB	TA	TB
C group	Positive	3/32	1/84	1/32	3/84	0/32	0/84	0/32	0/84
	Negative	24/32	67/84	4/32	11/84	0/32	2/84	0/32	0/84
SCM group	Positive	6/104	3/160	5/104	8/160	1/104	3/160	0/104	1/160
	Negative	59/104	95/160	31/104	38/160	2/104	8/160	0/104	4/160

Table 4. Data concerning the evaluation of CMTb and TESb scores performed in C and SCM groupep based on SCCb. Legend: SCCb – somatic cells count 15 days after parturition; CMTb – california mastitis test 15 days after parturition; TA – treatment A (600 mg of Cloxacillin and 300 mg of Ampicillin); TB – treatment B (375mg of Benzathine Cephalexin); C group – control group; SCM group – subclinical mastitis group.

SCCb (cell/ml)	CMTb	TESb score 1		TESb score 2		TESb score 3		TESb score 4	
		TA	TB	TA	TB	TA	TB	TA	TB
C group	Positive	1/136	1/244	4/136	0/244	0/136	0/244	0/136	0/244
	Negative	72/136	120/244	23/136	38/244	2/136	7/244	0/136	2/244
SCM group	Positive	6/136	9/244	5/136	5/244	1/136	3/244	0/136	2/244
	Negative	13/136	36/244	9/136	17/244	0/136	3/244	0/136	1/244

Table 5. Results concerning changing in CMTa *vs* CMTb for C and SCM group and the information about treatments received (TA *vs* TB). Legend: SCCa – somatic cells count at inclusion time (dry-off); CMTa – california mastitis test at inclusion time (dry-off); CMTb – california mastitis test 15 days after parturition; TA – treatment A (600 mg of Cloxacillin and 300 mg of Ampicillin); TB – treatment B (375mg of Benzathine Cephalexin); C group – control group; SCM group – subclinical mastitis group.

	Treatment at dry-off	CMTa-neg *vs* CMTb-neg	CMTa-neg *vs* CMTb-pos	CMTa-pos *vs* CMTb-neg	CMTa-pos *vs* CMTb-pos
C group	TA	27	2	1	2
	TB	74	4	6	0
SCM group	TA	69	5	23	7
	TB	121	10	24	5

Table 6. Table of contingency for the estimation of antibiotic treatment effect. The data refer to the number of treated teats. A) C cows; B) SCM cows.

A) P=0.297							
Observed				Expected			
	TA	TB	Total		TA	TB	Total
Positive	3	6	9	Positive	4	5	9
Negative	29	78	107	Negative	28	79	107
Total	32	84	116	Total	32	84	116
B) P< 0.001							
Observed				Expected			
	TA	TB	Total		TA	TB	Total
Positive	30	29	59	Positive	23	36	59
Negative	74	131	205	Negative	81	124	205
Total	104	160	264	Total	104	160	264

Table 7. Effect of the different antibiotic on teat in a preventive (expressed as % of teats that remain negative after treatment) and therapy (expressed as % of teats that become negative after treatment).

	TA	TB	SE	P-value
Preventive effect in “C cows”	92.85 %	94.91 %	6.08	0.760
Preventive effect in “SCM cows”	92.68 %	92.37 %	4.14	0.770
Therapeutic effect in “C cows”	50.00 %	100.00 %	14.56	0.022
Therapeutic effect in “SCM cows”	76.61 %	81.60 %	15.84	0.990