INTERPRETIVE SUMMARY

Randomized noninferiority clinical trial evaluating 2 commercial dry cow mastitis preparations. By Ospina et al. The study objective was to compare the efficacy of two commercial dry cow mastitis products (cephazoline and cephalonium dehydrate) at the quarter level and evaluate the cure risk, prevention of new infections during the dry period, prevalence of intramammary infections (IMI) after calving, and risk for a clinical mastitis case between calving and 60 days in milk (DIM). No difference was observed in efficacy between the 2 products evaluated when assessing the aforementioned quarter-level outcomes.

Noninferiority trial comparing two first generation cephalosporin at the dry off: effect of treatment on risk of cure

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ABSTRACT

The study objective was to compare the efficacy of two commercial dry cow mastitis products at the quarter level and evaluate the cure risk, prevention of new infections during the dry period, prevalence of intramammary infections (IMI) after calving, and risk for a clinical mastitis case between calving and 60 days in milk (DIM). A total of 590 cows (2,360 quarters) from 8 commercial dairy herds in Italy were enrolled and randomized to 1 of the 2 treatments at dry-off: Cefovet A (CF; 250 mg of cephazoline; Merial Italia SPA, Milano Italia), and Cepravin A (CP; 250 mg of cephalonium dihydrate MSD Animal Health Srl, Segrate Italia). Quarter milk samples were collected before dry cow therapy treatment at dry-off, 2 to 9 DIM, and 10 to 17 DIM and quarter samples of clinical mastitis cases were collected during the first 60 DIM. Noninferiority analysis was used to evaluate the effect of treatment on the risk of a bacteriological cure during the dry period, the primary outcome. The risk of developing a new intramammary infection during the dry period or the postpartum period, and the risk of a clinical mastitis event within 60 DIM was evaluated with multivariable logistic regression.

The overall crude quarter-level prevalence of infection at dry-off was 15.3%. The most common pathogen isolated from milk samples at dry-off was coagulase-negative *Staphylococcus*. Noninferiority analysis showed no effect of treatment on risk for a cure between dry-off and 2 to 9 DIM [least squares means (LSM): CF = 0.92 (95% CI 0.82 – 0.96), and CP = 0.93, 95% CI 0.86 – 0.97) and secondary analysis showed no effect of treatment on risk for presence of a new IMI at 2 to 9 DIM (LSM:CF = 0.09 (95% CI 0.06 – 0.13), and CP= 0.07 (95% CI 0.05 – 0.1)), nor was there a difference in risk of experiencing a clinical mastitis event between calving and 60 DIM (Hazard Ratio = 1.2, 95% CI 0.8 – 1.9). In conclusion, no difference was observed in efficacy between the 2 products evaluated when assessing the aforementioned outcomes.

Keywords: dry off therapy, noninferiority trial, first generation cephalosporin, intramammary infection

INTRODUCTION

For more than 60 years, antibiotics have been used to dry off animals in order to combat mastitis. This has helped to reduce the new infection risk from 30 to 60% in untreated cows down to 0 to 15% in treated cows (Berry and Hillerton, 2002). The dry period is a critical time point when lactating cows go through physiological changes to prepare the mammary gland for the next lactation. The importance of intramammary infections (IMI) during the dry period has been explored by several authors (Oliver and Mitchell, 1983; Eberhart, 1986; Erskine, 2001). Although self-cure, therapy or culling may reduce the amount of infected quarters; many more factors tend to increase the risk of new intramammary infection (**NIMI**), particularly during the beginning and the end of the dry period, i.e. involution and colostrogenesis (Bradley & Green, 2001). Persistence of preexisting IMI through the dry period and development of NMIM during the dry period are two important factors that increase the risk for manifestation of clinical mastitis in the next lactation.

Dry-cow therapy is regarded as one of the most important components of a mastitis control program, mainly because of the reduction in the number of staphylococcal and streptococcal infections (Whist et al., 2006), however, there is no data available for the prevalence of mastitis pathogens, cure risk, and prevention of new infections in Italian herds. The majority of NIMI are subclinical during the dry period, but can flare up as clinical mastitis, usually in early lactation

(Green et al., 2002). It has been estimated that 55% of environmental infections established early in the dry period persist into the next lactation and can possibly cause clinical flare-ups (Todhunter et al., 1995), and that 52% of all clinical coliform mastitis cases occurring in the first 100 d of lactation may originate during the previous dry period (Bradley and Green, 2000). Smith et al. (1985) also reported that the risk for NIMI from environmental pathogens can be 10 times higher during the dry period than during the lactation.

Blanket dry cow therapy (**DCT**), which refers to the intramammary infusion of all quarters of all cows at dry-off with a long-acting antibiotic, is a procedure recommended by the National Mastitis Council as a mastitis control practice, both for the purpose of curing existing subclinical infections and preventing new infections that could be acquired during the early dry period.

The study objective was to compare the efficacy of 2 commercial DCT products cephazoline; (CF); Cefovet A; Merial Italia SPA, Milano Italia and cephalonium dehydrate (CP) Cepravin, MSD Animal Health srl, Segrate Italia) as measured by quarter-level risk for cure of an IMI during the dry period, risk for development of NIMI over the dry period, risk for presence of an IMI postcalving, and risk for experiencing a clinical mastitis event between calving and 60 DIM. The hypothesis tested was that quarters infused with CF would have a noninferior proportion of quarters cured from preexisting IMI, and would have a similar presence of IMI postcalving, incidence of NIMI over the dry period, and incidence of clinical mastitis from calving to 60 DIM compared to quarters infused with CP.

MATERIALS AND METHODS

Study Design and product information

A randomized clinical trial to evaluate noninferiority between 2 DCT products was conducted from March 2014 to November 2014 in 8 commercial dairy herds in Italy (Figure 1). Cephazoline (CF, Cefovet A) contains 250 mg of cephazoline and is labeled to be effective on the treatment

and prevention of mastitis caused by *Streptococcus agalactiae Streptococcus uberis, Streptococcus dysgalactiae* and *Staphylococcus aureus*. Milk withholding times are 0 d after calving if dry period length is at least 30 d (if is shorter withholdingis 14 d); meat withholding time is 0 d with the exception of the mammary gland. The second antibiotic Cephalonium dehydrate (CP, Cepravin A) is composed of 250 mg cephalonium dihydrate. It is labeled to reduce the frequency of existing infections and prevent new infections caused by *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus disgalactiae, Streptococcus uberis, Actinomyces pyogenes, Corynebacterium ulcerans , Escherichia coli, Proteus spp., Klebsiella spp., Citrobacter spp. ed Enterobacter spp.* Milk withholding times are 51 d post infusion plus 168 h (7d) postcalving while meat withholding time is 2 d.

Herd Selection

Herds were considered for inclusion in the study if they agreed to comply with the study protocol and had regular DHIA testing. This convenience sample of herds averaged 450 lactating cows (range of 120 to 1,198), with an average bulk tank SCC of 240 10^3 cells/ mL (range of 180 to 350 10^3 cells/ mL), and a rolling herd average of 32 kg (range of 28 kg to 35 kg; Table 1). Herds A, B, C, D, E, F and G were located close to the laboratory at Dipartimento di Scienze Veterinarie per la Salute, la Produzione Animale e la Sicurezza Alimentare and herd F was close to Istituto Zooprofilattico Sperimentale del Lazio e della Toscana. All herds routinely used an internal sealant at dry off and a blanket DCT. All but one herd used Orbeseal (Zoetis Italia S.r.l., Latina , Italy), farm G used a different product, Intraseal (Norbrook Laboratories Limited, Newry, Northern Ireland).

Cow Enrollment

Cows eligible for enrollment were in good general health, had not received parenteral or intra-mammary treatment with an antibacterial or anti-inflammatory medication during a 30 days immediately before dry off and showed no signs of clinical mastitis on the day of dry-off. Cow identification numbers were previously assessed and animals were checked for previous medication. The authors residing at the local Italian Universities visited the herds weekly and conducted all study enrollment and aseptic sampling at the 3 different time points (dry-off = Time 0, 2 to 9 DIM = Time 1, and 10 to 17 DIM = Time 2). Aseptic milk samples from cows with clinical mastitis up to 60 DIM were collected by farm personnel.

Eligible cows were randomly allocated to treat all 4 quarters with CF or CP according to a previously prepared randomized spreadsheet created in Excel software (2010; Microsoft Corp., Santa Rosa, CA). Randomization was blocked within farms.

Cow data on calving, parity, clinical disease (including retained placenta, endometritis, metritis, lameness, abortion, clinical mastitis until 60 DIM and metabolic diseases such as ketosis and displaced abomasum) and culling was collected for all cows in the herd. During the study, Italian DHIA testing was performed monthly on all herds which evaluated milk production, fat, protein and SCC. Cow data were collected using a computerized herd record keeping system Afifarm (TDM SRL, San Paolo Brescia, Italy) or with an excel file.

Sample collection

Cows due to be dried off were brought into the parlor for their last milking and routine dry cow protocol. Teat end scores and cow hygiene were evaluated by university personnel. Teat end scores ranged from 1 to 4 according to the scale presented by Falkenberg et al., 2003; and udder

hygiene scores ranged from 1 (clean) to 4 (dirty) according to the scale presented by Schreiner and Ruegg, 2003. The udder and milk were inspected for signs of clinical mastitis (redness, swelling, pain). Before sampling, teat ends were carefully cleaned and disinfected with chlorhexidine then 70% alcohol. First streams of fore-milk were discharged, and then approximately 10 mL of milk was collected aseptically from each teat into sterile vials. These vials were previously identified with herd, cow number, quarter and date. Samples were stored at 4°C until bacteriological assays and SCC tests were performed. The milking took place after the milk sample was collected. Same procedure was performed for all milk samples obtained, i.e., at 2 to 9 DIM, at 10 to 17 DIM and when samples of clinical mastitis cases were collected by farm personnel. The clinical mastitis samples were frozen (-20º C) at the farm until the next visit by investigators (routinely within 1 week).

Immediately after the final milking, at dry-off, all 4 quarters were cleaned with a gauze soaked in 70% alcohol, then the assigned treatment was infused into each of the 4 quarters. Lastly, an internal teat sealant was infused. All cows were post-dipped and moved into dry cow facilities.

Bacteriological analysis

Bacteriological cultures were performed according to standards of the National Mastitis Council (NMC, 1999). Ten microliters of each milk sample were spread on blood agar plates (5% defibrinated sheep blood). Plates were incubated aerobically at 37°C and examined after 24 h. Colonies were provisionally identified on the basis of Gram stain, morphology, and hemolysis patterns, and the numbers of each colony type was recorded. Representative colonies were then sub-cultured on blood agar plates and incubated again at 37°C for 24 h to obtain pure cultures. Catalase and coagulase production were tested for gram-positive cocci. Gram negative isolates

were identified using colony morphology, Gram-staining characteristics, oxidase, and biochemical reactions on MacConkey's agar.

Definition of infection status

Presence of an IMI. An IMI was defined as 1 or more colonies isolated from a 0.01-mL milk sample for all pathogens except for CNS and *Bacillus* spp. For CNS, 2 or more colonies isolated from a 0.01-mL milk sample were needed to establish presence of an IMI (Dohoo et al., 2011).

Bacteriological cure. A cure was defined as the failure to culture pathogens originally present at the dry-off sample (Godden et al., 2003). Quarters that became compromised during dry period (e.g., blind quarters) or quarters with contaminated or missing samples were not included in the analysis.

New IMI. A new IMI was defined as quarter from which no pathogens were cultured at dry-off, but growth was detected in the first postpartum sample or if a different (new) pathogen was recovered in this sample. It was possible for the same quarter to experience both a cure and a new IMI. Quarters that had contaminated samples were not included in the cure analysis.

Statistical analysis

In order to estimate the sample size, the primary outcome was considered risk for a cure. The minimum difference in cure rate to declare noninferiority of CF compared with CP was prestated at 10%. To demonstrate noninferiority, a total of 550 cows (i.e., 275 cows; 1100 quarters per group) were estimated to be required assuming $\alpha = 0.05$, $\beta = 0.1$, a 10% loss to follow-up, and 30% of quarters infected at dry off (Blackwelder WC. "Providing the Null Hypotheses" in Clinical Trials. Control Clin. Trials. 1982; 3:345-353)

All statistical analyses were conducted using SAS (version 9.3: SAS Institute Inc., Cary, NC).Initially, descriptive statistics and plots were generated for exploratory data analysis. Basic diagnostic techniques were used to evaluate normality and presence of outliers for continuous data. Characteristics of cows and quarters assigned to the 2 treatment groups were compared at dry-off in univariate analysis using the PROC FREQ (chi-squared test) for categorical variables and PROC TTEST for continuous variables.

Noninferiority analysis of the effect of treatment on the risk difference for bacteriological cure was completed by comparing the risk difference to a noninferiority margin (Miettinen and Nurminen 1985, Farrington and Manning 1990,) with PROC FREQ (Schuirmann 1999, Dann and Koch 2008).

The effect of treatment on the odds of cure and new IMI during were evaluated using PROC GLIMMIX with herd and cow included as random effects to account for clustering effects of cows within herds, and quarters within cows. The covariates offered to the model were: DCT (CP or CF), parity group (2 levels; lactation = 1, lactation > 2), previous lactation linear score, previous lactation total milk (kg), dry period length, body condition score at dry off (d; 2 levels $\leq$ 3.0 and > 3.0), teat end score at dry off (2 levels; teat end score 1 and 2 = 1, teat end score 3 and 4 = 2), hygiene score at dry off (2 levels; hygiene score 1 and 2 =1, hygiene score 3 and 4 = 2).

Time to event analysis was performed with cox proportional hazards regression (PROC PHREG). Clustering at the herd level was controlled for with a COVSANDWHICH statement. This was used to describe the effect of dry cow treatment on experiencing a case of clinical mastitis between calving and 60 DIM, no cows were reported to have calved with clinical mastitis. The failure date was defined as the date when the quarter was reported to have clinical mastitis, those quarters that did not experience a mastitis event were classified as censored if the cow was culled, or dead $\leq$ 60

DIM. The covariates included in the model were: DCT (CP or CF), parity group (2 levels; lactation = 2, lactation $\geq$ 2), previous lactation linear score, previous lactation total milk (kg), dry period length (d), teat end score at dry off (2 levels; teat end score 1 and 2 = 1, teat end score 3 and 4 = 2), hygiene score at dry off (2 levels; hygiene score 1 and 2 =1, hygiene score 3 and 4 = 2). During the model building process, models were compared using -2 log-likelihood statistics and the final model fit was assessed plotting deviance residuals.

Results

A total of 2,360 quarters (590 cows) were enrolled in the study between March and November of 2014. Of those, 1,196 and 1,164 quarters were allocated to treatment groups CF, and CP, respectively. Figure 2, describes quarters lost to follow-up. The treatment groups did not differ at enrollment regarding the cow-level parameters (Table 2). There was no significant difference in milk production (kgs) in the previous lactation (P = 0.3), the mean ± standard deviation were 11,149 ± 3,607; 10,920 ± 3,314 for CF and CP groups, respectively.

IMI Status at Dry-Off

A total of 2,238 quarters were used evaluated for subclinical infection at dry-off. The overall crude prevalence of IMI at dry-off was 15.3% (Table 3) and was not different among treatments P = 0.4, odds ratio of an infection based on treatment was 0.9 (95% CI: 0.7 – 1.1). The pathogen most commonly isolated from milk samples at dry off was CNS, representing 63% of all isolates recovered (Table 4).

Noninferiority analysis and the effect of treatment on odds of experiencing a cure between dry-off and post-calving

A total of 347 quarters had an IMI present at dry-off and were at risk for cure Table 4. Noninferiority analysis showed that there was no difference between the risk of cure between the treatment groups. The proportion difference was 0.01, the apriori set limit was 0.1. The 90% CI for proportion difference was -0.04 to 0.07, P < 0.0001. The interpretation of these results is that the null hypothesis that CF is inferior to CP is rejected. There was no significant difference in the odds of experiencing a cure based on treatment (P = 0.7) and relevant covariates (P > 0.6; Table 5). The least square means (LSM) of the treatment groups were 0.92; 95% CI: 0.82 to 0.96 for CF and 0.93; 95% CI 0.86 to 0.97 for CP.

Effect of treatment on Odds for Presence of a NIMI at 2 to 9 DIM

A total of 2,015 quarters were used in the analysis of the odds of presence of infection at 2 to 9 DIM. The overall crude proportion of infections was 7.7%, with no difference among treatment groups (P = 0.13); in addition to treatment, the following variables were included in the model: herd, parity group, hygiene at dry-off, length of dry period, milk production at lactation, linear score at dry off, teat end score at dry-off, presence of infection at dry off (Table 6).The most common pathogen isolated was CNS (60% of all isolates recovered; Table 7).

Effect of treatment on risk of experiencing a clinical mastitis event between calving and 60 DIM

The Cox proportional hazards model was used for the analysis of the risk of a clinical mastitis case at the cow level. There were 568 cows available for analysis but only 521 cows used due to missing information. In these 521 cows, there were 75 cases of clinical mastitis within 60 DIM. There was no significant difference based on treatment (P = 0.3; Table 8). The most common pathogen isolated was *Escherichia coli* 23% (5 cases from CF, 10 from CP), 25% of the samples submitted yielded no growth.**Discussion**

There was no significant difference between CF and CP in the cure risk of infections present at dry-off when quarters were re-evaluated at 2 to 9 DIM. Additionally, there was no significant difference when the risk of NIMI at 2 to 9 DIM, or clinical mastitis events within 60 DIM were compared between the two products.

This is the first prospective multi-region, multi-herd non-inferiority study performed in Italy. It is difficult to compare results from this study to previous research because no such study has

been performed in Italy or other countries with these two drugs. ??Have there been other studies that looked at these drugs in other ways???

One of the major strengths of this study is that it was conducted in commercial herds from different regions of Italy, using different dry cow housing and management strategies. Also, although the study was performed in Italy, the types and frequencies of pathogens recovered were similar to those reported in other North American dry cow mastitis studies (Arruda et al 2013a, Arruda et al 2013 b). However, it is important to note that these herds were larger than average herds in Italy, where the average herd size is _____ (reference for average herd size in Italy). These herds also had higher than average daily milk production, and average SCC. … more information about how these herds compared to average Italian herds… for example: management, housing of cows… etc.

Effect of treatment on risk of cure between dry-off and 2 to 9 DIM

The current study found that CF was noninferior to CP on the risk of experiencing a cure of an IMI during the dry period. The crude proportion of quarters experiencing a cure in this study (89.6%) was similar to previous North American studies (Godden et al., 2003; Pantoja et al., 2009; Gundelach et al., 2011; Arruda et. al., 2013). ARE THERE OTHER STUDIES THAT LOOKED AT THESE DRUGS WITH A CONTROL?? If there are, compare those cure results with these…

It is important to discuss that the crude number of IMI at dry-off was lower than expected ~15%, instead of the anticipated ~30%, therefore fewer quarters were at risk for a cure. A post-hoc power calculation estimated that the study had approximately ___ power to detect a difference (delta) of 10% in cure risk between treatment groups compared. Although there was a loss of

power due to the smaller N, we do not consider this a weakness in the study given that the numeric difference observed in cure risk was very small (observed delta = 0.01). Therefore, we do not believe that the loss of power in any way compromised the validity of the conclusions reached in this study.

IMI Status at dry-off, effect of treatment on risk of a NIMI 2 to 9 DIM, and clinical mastitis infections

The current study found no effect of treatment on risk for presence of a NIMI 2 to 9 DIM. The crude prevalence of infection at dry-off in this study (15.3%) was slightly lower than North American studies, but within the range (Godden et al., 2003; Pantoja et al., 2009; Gundelach et al., 2011; Arruda et. al., 2013). Some difference may be due to differences in IMI definitions and sampling methodology among studies. Godden et al., for example, defined an IMI infection as the presence of 1 colony in ten microliters for any pathogen, whereas Pantoja et al., reported a 12.8% prevalence, but the threshold for a NIMI was 3 or more colonies in the same amount of milk. The IMI prevalence post-calving can be variable and can range from 6.9 to 40.4% (Pantoja et al., 2009; Hallberg et al., 2006). This high variability is likely due to differences in population, e.g., the Hallberg et al., study enrolled only high somatic cell count cows. The postcalving prevalence of IMI in this study at 2 to 9 DIM was 7.8%.

Similar to previous dry cow mastitis studies (Godden et al., 2003; Pantoja et al., 2009; Gundelach et al., 2011; Arruda et. al., 2013), the pathogen most commonly isolated was CNS. The second most common pathogen isolated was *E. coli*, and *Strep. dysgalactiae*, both in much smaller numbers that CNS. Similar to the Arruda et al., paper, *Bacillus* spp. was found at all culture time points. Although the role of *Bacillus* spp. is not well understood, and may not be reported

regularly, it has been shown to be the cause of clinical mastitis (Nieminen et al., 2007). In this study, there was only 2 cases of clinical mastitis where *Bacillus* spp. was cultured in the milk sample.

Although there was no effect of treatment on the risk for development clinical mastitis, there was a numerical difference between the treatment groups (41% vs. 59%). The crude incidence of clinical mastitis (10%) in this study is higher than other reported studies from North America which report 3 to 6% (Godden et al., 2003; Gundelach et al., 2011, Arruda et al., 2013). The results of the cultures are similar to other studies, where no growth was the most likely outcome, followed by *E. coli*. NEED TO INCLUDE MORE INFORMATIONHERE ABOUT WHY WE SAW more MASTITIs that other studies…

Secondary findings

The current study did not detect any statistically significant associations with other covariates, with the exception of length of dry period and teat score at dry off. However, the change in the odds associated with a 1 unit increase in either of these parameters was negligible. The lack of significant secondary findings may be associated with lack of power because the sample size was calculated based on the cure risk, additionally, we had fewer cases than expected. Most of these secondary findings were based on subjective interpretation and this may not have been a sensitive enough tests to detect differences among groups.

Conclusions

Results from this noninferiority study demonstrate that in herds using a blanket teat sealant infusion at dry-off, no difference in efficacy exited between the products CF and CP regarding the cure risk for an existing infection at dry-off, odds of developing a new infection 2 to 9 DIM, and

risk of clinical mastitis event within 60 DIM. As such, concerns about differences in product efficacy can be put aside. In an effort to reduce the risk of residues in milk or tissue, the use of CF, which has a 0 milk and tissue withhold if the dry period length is at least 30 d, can be considered as a noninferior DCT alternative to CP.

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