Randomized noninferiority field trial evaluating cephapirin sodium for treatment of nonsevere clinical mastitis

T. Tomazi,* T. A. F. Lopes,† V. Masson,† J. M. Swinkels,‡ and M. V. Santos*¹

*Department of Animal Nutrition and Production, School of Veterinary Medicine and Animal Science, University of São Paulo, Pirassununga 13635-900, São Paulo, Brazil

†MSD Animal Health, São Paulo, 04583-110, Brazil

‡MSD Animal Health, Global Ruminants Business Unit, Boxmeer, 5830 AA, the Netherlands

ABSTRACT

The general objective of this study was to evaluate whether cephapirin sodium is noninferior compared with a positive control broad-spectrum product formulated with a combination of antimicrobials for intramammary treatment of nonsevere clinical mastitis. In addition, we compared the efficacy of treatments on the cure risks of pathogen groups (gram-positive, gram-negative, and cultures with no growth) based on culture results. A total of 346 cows distributed in 31 commercial dairy herds were selected to participate in the study, although only 236 met the criteria for evaluation of microbiological cure. Coagulase-negative staphylococci were the most isolated gram-positive pathogens in pretreatment milk samples, whereas the most common gram-negative bacterium was *Escherichia coli*. Cows attending the postadmission criteria were treated with 4 intramammary infusions (12 h apart) of one of the following antimicrobials: 300 mg of cephapirin sodium + 20 mg of prednisolone (CS), or the positive control treatment formulated with a combination of antimicrobials (200 mg of tetracycline + 250 mg of neomycin + 28 mg of bacitracin + 10 mg of prednisolone; TNB). Noninferiority analysis and mixed regression models (overall and considering the pathogen groups) were performed for the following outcomes: bacteriological cure (absence of the causative pathogens in cultures performed in milk samples collected at 14 and 21 ± 3 d after enrollment), pathogen cure (absence of any pathogen on both follow-up samples), clinical cure (absence of clinical sign in the milk and mammary gland at 48 h after the last antimicrobial infusion), extended clinical cure (normal milk and normal gland on the second posttreatment sample collection (d 21), and linear score of somatic cell count cure [linear score of somatic cell count recovery (≤ 4.0) on d 21 ± 3 after enrollment]. No significant differences

were observed between treatments regarding any of the evaluated outcomes in both regression models (overall and considering the pathogen groups). Noninferiority of CS relative to TNB was inconclusive for bacteriological cure (CS = 0.68; TNB = 0.73) and clinical cure (CS = 0.88; TNB = 0.94), as the confidence intervals crossed the pre-stated margin of noninferiority ($\Delta = -0.15$). Cephapirin sodium was noninferior compared with TNB for pathogen cure (CS = 0.36; TNB = 0.35), extended clinical cure (CS = 0.93; TNB = 0.92), and linear score of somatic cell count cure (CS = 0.29; TNB = 0.28). In conclusion, the use of intramammary CS for treatment of nonsevere clinical mastitis has similar efficacy as a treatment regimen with a combination of antimicrobial agents (tetracycline + neomycin + bacitracin), although noninferiority analysis showed inconclusive results for bacteriological and clinical cures.

Key words: antimicrobial, clinical mastitis, intramammary treatment, noninferiority analysis

INTRODUCTION

Clinical mastitis is a common disease affecting dairy cattle and one of the most important causes of antimicrobial consumption in dairy herds (Mitchell et al., 1998; Saini et al., 2012). Along with mastitis prevention measures (e.g., maintenance of cows' hygiene and proper milking management), the use of effective antimicrobials to eliminate mastitis-causing pathogen and to recover milk production as quickly as possible are needed for reduction of financial losses caused by clinical mastitis (CM).

Several antimicrobials have been used for treatment of CM, especially those administered by the intramammary route (Pol and Ruegg, 2007). However, considering the current worldwide demand for prudent use of antimicrobials in production animals (Van Boeckel et al., 2017), it is in the best interest of the dairy industry not only to reduce antimicrobial use, but also to use the most efficient products for treatment of CM in dairy cows. The use of more efficient antimicrobials for rapid

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¹Corresponding author: mveiga@usp.br

elimination of mastitis causing pathogens can result in less damage to the mammary gland, thus reducing the time of cows' recovery.

Antimicrobials of the cephalosporin class are among the compounds with the highest frequency of use for treatment of IMI in dairy herds (Pol and Ruegg, 2007; Stevens et al., 2016). Currently, there are 4 generations of cephalosporins, which have evolved from compounds with narrow spectrum, such as the first-generation cephalosporins (i.e., better activity against gram-positive pathogens; Pfeifer et al., 2010), to compounds with a wider spectrum as fourth-generation cephalosporins, which have good activity against both gram-positive and gram-negative microorganisms (Schukken et al., 2013). Cephalirin sodium (**CS**) is a first-generation cephalosporin antimicrobial frequently used for treatment of gram-positive mastitis (Pol and Ruegg, 2007; Apparao et al., 2009), although it has limited antimicrobial activity against gram-negative microorganisms (Constable and Morin, 2002). In a previous study in the United States, CS was noninferior to ceftiofur hydrochloride with regard to bacteriological cure of gram-positive cases, and for clinical cure of all cases; in addition, CS showed a significantly lower bacteriological cure in gram-negative cases than ceftiofur (Schukken et al., 2013).

Several studies evaluating the efficacy of antimicrobials for treatment of CM were conducted as randomized trials comparing the efficacy of an antimicrobial product with an untreated control (Roberson et al., 2004; Schukken et al., 2011) or evaluating 2 or more treatment protocols to determine whether one product was superior to other (Deluyker et al., 1999; Bradley and Green, 2009; Cortinhas et al., 2016). However, few studies have reported noninferiority trials for comparison of a specific antimicrobial with a positive control (Schukken et al., 2013; Bryan et al., 2016; Vasquez et al., 2016). Noninferiority trials can be designed when particular factors can influence the choice of treatment, such as lower cost, efficiency over certain groups of pathogens, fewer adverse effects, reduced frequency of administration, or availability of antimicrobial products in the market.

In this study, we used as a positive control an approved intramammary product with broad spectrum, comprising a combination of tetracycline + neomycin + bacitracin, which is highly marketed in Brazil for treatment of CM (T. Tomazi and M. V. dos Santos, unpublished data), mainly because it has a broad spectrum of antibacterial activity. However, when nonsevere CM is mainly caused by gram-positive pathogens, the use of broad spectrum antimicrobials may not be advantageous over a compound with narrow spectrum, which can eventually be purchased at a lower cost, and 2 fac-

tors can reinforce this statement: (1) cases with negative culture results (no growth) represent 20 to 50% of CM cases (Ruegg, 2012), and so antimicrobial therapy would not be justified for those cases; and (2) most nonsevere CM caused by gram-negative bacteria are spontaneously cured without using any antimicrobial (Lago et al., 2011). Therefore, in herds where blanket treatments are performed, the use of narrow-spectrum antimicrobials may not be less effective compared with a broad-spectrum product for treatment of nonsevere CM.

The main objective of the present study was to evaluate whether CS was noninferior compared with another commercial intramammary compound with broad spectrum formulated with a combination of antimicrobials (tetracycline + neomycin + bacitracin) for treatment of nonsevere CM. In addition, a secondary objective was to compare the efficacy of treatments on cure risks by stratification of CM cases in pathogen groups based on culture results.

MATERIALS AND METHODS

Study Design

This was a randomized noninferiority trial comparing 2 commercial antimicrobials for treatment of nonsevere CM. The null hypothesis (H_0) was that CS was inferior to the reference product (combination of tetracycline, neomycin, and bacitracin; **TNB**), and the alternative hypothesis (H_A) was that CS was not inferior to the TNB (positive control treatment) by more than the pre-stated noninferiority margin (Δ). These hypotheses statements can be summarized by the following formulas:

$$H_0: [P_{\text{cure}}(\text{CS}) - P_{\text{cure}}(\text{TNB})] \leq -\Delta$$

$$H_A: [P_{\text{cure}}(\text{CS}) - P_{\text{cure}}(\text{TNB})] > -\Delta,$$

where P_{cure} is the probability of cure (bacteriological, pathogen, clinical, extended clinical, SCC recovery), and Δ is the noninferiority margin. Thus, by rejecting H_0 , we accept that the intramammary administration of CS is noninferior to the reference product in relation to CM treatment outcomes.

Sample Size Determination

Sample size was calculated using the Sealed Envelope Ltd. (2012) online software. Risk for a cure was considered the primary outcome for the a priori sample size calculation, which was performed using the confidence interval (**CI**; 95%) approach. A binomial distribution

was performed assuming a TNB cure risk of 75% based on a previous randomized clinical efficacy trial (Corrinhas et al., 2016). The minimum difference in cure risk to declare noninferiority of CS compared with TNB was 15% (i.e., $\Delta = 0.15$), which was based on other studies that also applied this value for noninferiority margin (Schukken et al., 2013; Vasquez et al., 2016). Therefore, to demonstrate noninferiority, 143 infected cows were needed in each group assuming $\alpha = 0.05$, $\beta = 0.1$, and $\Delta = 0.15$. Assuming 15% of loss to follow-up, at least 329 cows needed to be enrolled in this study.

Herd Selection

A convenience sample of 31 commercial dairy herds in Brazil, distributed in States of Minas Gerais ($n = 9$) and Paraná ($n = 22$), and with an average of 205 (± 149) lactating cows, were selected for this study. Herd selection was based on the willingness of owners to participate in the study, and on herd performance of exactly 2 daily milkings, with approximately 12 h interval between milkings for reduction of variation of treatment intervals.

Selection of Dairy Cows

Lactating cows were eligible for participation in the study when they (1) were in good general health condition, (2) had visible good teat integrity (i.e., smooth and healthy teat skin without lacerations or bruising, and teat end without rough callous ring, hyperkeratosis, or radial cracking), (3) had CM in only one mammary quarter on the day of identification, (4) were more than 25 d from anticipated dry-off date, (5) were not under undergoing treatment for any other illness, and (6) have not received systemic or intramammary antimicrobials or anti-inflammatory treatments within a 30-d period before the CM identification,

Clinical mastitis was defined as a quarter with abnormal milk, accompanied or not by other clinical signs, such as udder swelling, redness, heat, and pain (IDF, 1999). The severity of CM was defined according to the classification of Wenz et al. (2006): mild—changes only in the milk appearance, such as abnormal viscosity (watery appearance), color or consistency (presence blood, flakes or clots); moderate—presence of abnormal milk accompanied by changes in the affected udder (hardening, swelling, redness, or a combination of these); and severe—the combination of abnormal milk, with signs of inflammation in the udder and systemic signs (body temperature $>39.5^{\circ}\text{C}$, lack of appetite, dehydration, weakness, depression). Only cows with nonsevere CM were included in the study.

Postadmission Exclusion

The following criteria were used to exclude cows after enrollment in the study: (1) pretreatment milk sample was contaminated (>2 bacteria species isolated); (2) evolution to a severe case of CM (treatment failure); (3) occurrence of any other undergoing treatment for any other illness; (4) occurrence of CM in another quarter after enrollment; and (5) daily milk yield <10 kg/d based on the test day before the identification of the CM case.

Treatment Protocols and Randomization

Cows were randomly assigned to either of the 2 treatment protocols according to a previously prepared randomized spreadsheet using the RAND function of Excel software (2010, Microsoft Office Corporation, Redmond, WA). For each farm, a form was created with the list of randomized order of treatment protocols and the following information had to be filled before the beginning of each treatment: date of CM case, identification of affected cow and mammary quarter, and name of person responsible for treatment. Before the beginning of study, the form was placed in the milking parlor or in any other facility where the treatments were performed in the farm.

Both therapeutic protocols for treatment of CM were performed by 4 intramammary infusions of one of the following commercial antimicrobials into the affected quarter: CS group—product composed of 300 mg of cephapirin sodium + 20 mg of prednisolone (Mastiplan LC, MSD Animal Health, São Paulo, Brazil); and TNB group—product composed of 200 mg of tetracycline + 250 mg of neomycin + 28 mg of bacitracin + 10 mg of prednisolone (Mastijet Forte, MSD Animal Health, São Paulo, Brazil). For both protocols, treatments were performed at an interval of approximately 12 h.

Sample Collection

Training was performed with farm personnel on CM identification and on severity score classification before the beginning of the study. The collection of milk samples and recording of clinical data were performed by trained farm personnel on the day of CM identification (d 0) and at 14 ± 3 d (d 14) and 21 ± 3 d (d 21) after the onset of the treatment. For milk sampling, the first 3 streams of milk were discarded, and the teat end of the affected quarter was disinfected with a gauze soaked in 70% alcohol. A duplicate milk sample was collected to have a backup sample in case of contamination result in the initial culture. All collected milk samples were

immediately placed on ice and stored in freezers at the farm for less than 2 wk before microbiological culture.

Milk samples were also collected from mammary quarters with CM for SCC determination before milking of the affected cow at d 0 and 21. Milk samples for SCC determination were collected into plastic tubes (40 mL) containing Bronopol (Microtabs II, D&F Control Systems Inc., Norwood, MA) as a preservative (0.05 g/100 mL of milk) according to the International Dairy Federation (IDF, 1995). Quarter milk samples were kept refrigerated (1–8°C) and submitted for analysis of SCC within 3 to 5 d. The SCC was determined by flow cytometry using a fluoro-opto-electronic cell counting method (Somacount 300, Bentley Instrument Inc., Chaska, MN).

Bacteriological Analysis

Bacteriological culture was performed according to National Mastitis Council (1999). Briefly, 0.01 mL of milk was plated on blood agar and incubated aerobically for 24 and 48 h at 37°C. A milk sample was defined as negative if no colonies were observed on the streaking field of the blood agar plate after 48 h of incubation. On the other hand, a milk sample was defined as positive if at least one colony of any pathogen was observed in the streaking field of the blood agar plate. If 2 distinct colonies (based on the morphological features) were observed in the streaked field, the milk sample was defined as a mixed culture. Contamination was defined when more than 2 different colony types were present in the streaked field of the agar plate.

After observation of colony morphology and hemolytic patterns on blood agar, isolates were examined further by means of catalase test using hydrogen peroxide (3%), 3% KOH, and Gram staining of organisms. Additional biochemical and metabolic evaluations were performed as needed using specific microbiological tests.

Coagulase test using defibrinated rabbit plasma was performed to distinguish *Staphylococcus aureus* from CNS. Streptococci were defined as esculin-positive (*Streptococcus uberis* or *Streptococcus* spp.) or esculin-negative (*Streptococcus agalactiae* or *Streptococcus dysgalactiae*). The Christie, Atkins, Munch-Petersen test was used to distinguish *Strep. agalactiae* from *Strep. dysgalactiae*. The bile esculin test and the pyrrolidonyl arylamidase test (Probac do Brasil, São Paulo, Brazil) were used to differentiate isolates as *Streptococcus* spp., *Strep. uberis*, or *Enterococcus* spp.

Isolates with positive reaction in the potassium hydroxide (KOH) test were suggestive of gram-negative microorganisms, and then identified by colony morphology on MacConkey's agar. In addition, the fol-

lowing biochemical characteristics were evaluated for gram-negative microorganisms: sucrose and glucose fermentation, hydrolysis of urea, gas production, motility capacity, indole production, H₂S production, L-tryptophan deaminase, and lysine reaction.

Microbiological testing for identification of *Mycoplasma* spp. was not performed in this study.

Blinding

Blinding of the farm personnel at the time of treatment was not possible. However, laboratory personnel were blinded to treatment. In addition, both farm and laboratory personnel were unaware of objective outcomes, including clinical, bacteriological, and pathogen cure.

Assessment of Treatment Outcomes

Bacteriological cure, pathogen cure, and clinical cure were defined as primary outcomes. Extended clinical cure and posttreatment linear score of SCC (LSSCC) cure were defined as secondary outcomes.

Bacteriological Cure. A cow was considered bacteriologically cured if a microorganism was identified in the milk sample collected on d 0, and the same species was not isolated in any of milk samples collected posttreatment (d 14 or 21). If the same pathogen isolated on d 0 was identified in either of the posttreatment samples, the quarter was considered noncured. Only cows with culture results for posttreatment milk samples were included in this evaluation. In addition, samples with negative culture (no growth) on d 0 were not included in the analysis of bacteriological cure.

Pathogen Cure. A cow was identified as pathogen cured if both samples collected posttreatment had no growth, regardless whether the microbiological culture on d 0 also presented no growth (Vasquez et al., 2016). Cows with missing culture results, or with contaminated samples, were not considered for pathogen cure.

Clinical Cure. A cow was regarded as clinical cured if both milk and mammary gland had a normal appearance in the clinical examination performed approximately 48 h after the last antimicrobial infusion.

Extended Clinical Cure. If a cow had normal milk and normal gland on the second posttreatment sample collection (d 21), she was defined as extended clinically cured.

Linear Score of SCC Cure. Somatic cell counts were converted to linear scores by the following formula: LSSCC = $\log_2$ (SCC/100) + 3 (Schukken et al., 2003). Cows with LSSCC ≤ 4.0 ($\leq 200 \times 10^3$ cells/mL) in the milk sample collected on d 21 were considered LSSCC cured, regardless of whether the LSSCC at d

0 was higher or lower than 4.0. If the LSSCC on d 21 was >4.0 , the cow was considered noncured for LSSCC.

If no posttreatment samples were taken for reasons other than insufficient progress (e.g., death or culling), the cow was not eligible for bacteriological and pathogen cure evaluation, but she could still be considered for evaluation of clinical cure, extended clinical cure, or LSSCC cure if these data were available.

Statistical Analysis

Descriptive statistics and plots were generated for exploratory data analysis. Basic diagnostic techniques were performed to evaluate normality and homogeneity of variance using SAS version 9.4 (SAS Institute Inc., Cary, NC). Characteristics of cows and quarters assigned to the treatment groups were compared considering the day of cows' enrollment in univariate analysis using the PROC FREQ command of SAS version 9.4; chi-squared test was used for categorical variables (i.e., quarter position) and PROC TTEST was used for continuous variables (i.e., parity, DIM, and quarter-level LSSCC).

Noninferiority analysis of the treatment effects on the risk difference for binary outcomes were completed with 1-sided 95% CI and noninferiority margin (i.e., $\Delta = 0.15$) using PROC FREQ of SAS 9.4. The determination and interpretation of possible outcomes of noninferiority trial (noninferior, inconclusive, or inferior) were made as described by Piaggio et al. (2006).

In addition, the treatment effects on the primary and secondary outcomes were evaluated at the cow level as binomial response variables using 2 logistic regression models. Treatment effects on outcomes for the first model (model 1) were evaluated considering all enrolled CM cases, regardless of the culture results as following:

$$\begin{aligned} \text{logit (outcome)} = & \text{intercept} + \text{position} + \text{parity} \\ & + \text{DIM} + \text{treatment} + \text{herd (random)} + e, \end{aligned}$$

where outcome is cure risks (bacteriological, clinical, pathogen, extended clinical or LSSCC); position is the position (front or hind) of the affected quarter; parity is the number of lactations of enrolled cows (categorized as 1, 2, 3 or ≥ 4); DIM is the days since calving of the enrolled cow (categorized as 0–100, 101–200, 201–300, and ≥ 301); treatment is a binary variable indicating either CS or TNB treatment groups; herd is a set of indicator variables for farm included as a random effect; and e is the random residual error.

For the second model (model 2), the effect of pathogen group (gram-positive, gram-negative, and no growth) and its interaction with treatment was included in the previous model. Least squares means

were calculated from the models and used to evaluate difference between treatments (Schukken et al., 2013). For both regression models, analyses were carried out using PROC GLIMMIX of SAS version 9.4. Statistical significance was assumed at $P \leq 0.05$.

RESULTS

Descriptive Results

In total, 346 CM cases from 31 herds met the enrollment criteria. At the onset of treatments, no significant difference was present between groups regarding parity, DIM, pretreatment SCC linear score, and quarter position (Table 1). In addition, the distribution of treatments was not statistically different ($P = 0.18$); 51.2% ($n = 177$) of the cows were assigned to the CS group, and 48.8% ($n = 169$) were assigned to the TNB group.

A total of 110 enrolled cows (63 in the CS group and 47 in the TNB group) were not evaluated for microbiological cure (bacteriological or pathogen), and the frequency of not enrolled cases were statistically similar among groups. The reasons for cow-cases postadmission exclusion were as follows: occurrence of mastitis in another quarter postenrollment ($n = 50$; 30 cows in CS group, and 20 in TNB group; $P = 0.13$); secondary treatment of the affected quarter (treatment failure) or treatment of the cow because of other reasons ($n = 30$; 18 cows in CS group, and 12 in TNB group; $P = 0.23$); postadmission samples not collected for microbiological culture ($n = 18$; 9 cows in CS group, and 9 in TNB group; $P = 0.89$); loss of the samples ($n = 6$; 2 cows in CS group, and 4 in TNB group; $P = 0.47$); contamination of both pretreatment milk samples collected in duplicate ($n = 5$; 3 cows in CS group, and 2 in TNB group; $P = 0.60$); and culling of the cow (1 cow from CS; $P = 0.30$). Therefore, 236 cow-cases of CM were evaluated for bacteriological or pathogen cure.

Twenty-six cows did not have cases enrolled for microbiological cure, but they still were assessed for clinical cure ($n = 26$), extended clinical cure ($n = 18$), and LSSCC cure ($n = 2$).

After cow-cases postadmission exclusion, for all evaluated outcomes, no differences ($P > 0.26$) were observed between groups for parity, DIM, pretreatment SCC linear score, and quarter position.

Pretreatment Bacteria Identification

The frequencies of bacteria isolation in the pretreatment cultures were evenly distributed ($P > 0.05$) between treatment groups. Therefore, of the 236 cow-cases not excluded postadmission, 71 (30.1%) had no bacteria growth after 48 h of incubation at 37°C. A total of 130

Table 1. Descriptive results of pretreatment cow-level characteristics (mean and SD in parentheses) and quarter position (number of occurrences and percentage in parentheses) according to intramammary treatment group

Item	Treatment		P-value
	CS ¹ (n = 177)	TNB ² (n = 169)	
Cow characteristics, no. (SD)			
Parity	3.1 (1.6)	3.0 (1.6)	0.70
DIM ³	180 (144)	197 (143)	0.98
SCC linear score ⁴	7.5 (2.0)	7.4 (2.2)	0.46
Quarter position, no. (%)			
Front (n = 202)	101 (50.0)	101 (50.0)	0.61
Hind (n = 144)	68 (47.2)	76 (52.8)	

¹Tested antimicrobial: 300 mg of cephalixin sodium + 20 mg of prednisolone.

²Positive control antimicrobial: 200 mg of tetracycline + 250 mg of neomycin + 28 mg of bacitracin + 10 mg of prednisolone.

³Days in milk at the day of clinical mastitis occurrence.

⁴Linear score of SCC (LSSCC) measured at the day of clinical mastitis identification; calculated as LSSCC = $\log_2(\text{SCC}/100) + 3$ (Schukken et al., 2003).

(55.1%) cases had isolation of gram-positive bacteria, and only 29 (12.3%) had isolation of gram-negative bacteria. In addition, another 6 (2.5%) cases were identified as nonbacterial microorganisms (yeast or *Prototheca* spp.). Among gram-positive bacteria, CNS was the most frequently isolated microorganism (25.4%; 33/130), followed by *Staphylococcus aureus* (17.7%; 23/130) and *Streptococcus uberis* (16.9%; 22/130). On

the other hand, *Escherichia coli* (72.4%; 21/29) was the gram-negative pathogen with the highest frequency of isolation (Table 2).

Bacteriological Cure

Out of 236 cases that attended the inclusion criteria, 71 had no culture growth in the pretreatment milk

Table 2. Frequency of bacteriological culture results of milk samples collected at the time of detection (pretreatment) of nonsevere clinical mastitis (CM), and the intramammary treatment regimen (cephalexin sodium vs. tetracycline + neomycin + bacitracin) to which the cases were submitted

Microorganism	CS ¹		TNB ²		Total	
	No.	%	No.	%	No.	%
No growth	29	12.3	42	17.8	71	30.1
Gram-positive	65	27.5	65	27.5	130	55.1
CNS	17	7.2	16	6.8	33	14.0
<i>Staphylococcus aureus</i>	15	6.4	8	3.4	23	9.7
<i>Streptococcus uberis</i>	7	3.0	15	6.4	22	9.3
<i>Corynebacterium</i> spp.	10	4.2	10	4.2	20	8.5
<i>Streptococcus</i> spp.	3	1.3	8	3.4	11	4.7
<i>Streptococcus agalactiae</i>	7	3.0	2	0.8	9	3.8
<i>Streptococcus dysgalactiae</i>	4	1.7	4	1.7	8	3.4
<i>Bacillus</i> spp.	1	0.4	2	0.8	3	1.3
<i>Trueperella pyogenes</i>	1	0.4	—	—	1	0.4
Gram-negative	17	7.2	12	5.1	29	12.3
<i>Escherichia coli</i>	12	5.1	9	3.8	21	8.9
<i>Serratia</i> spp.	3	1.3	1	0.4	4	1.7
<i>Klebsiella</i> spp.	1	0.4	1	0.4	2	0.8
<i>Enterobacter</i> spp.	—	—	1	0.4	1	0.4
<i>Pseudomonas</i> spp.	1	0.4	—	—	1	0.4
Other pathogen	3	1.3	3	1.3	6	2.5
Yeast	2	0.8	2	0.8	4	1.7
<i>Prototheca</i> spp.	1	0.4	1	0.4	2	0.8
Total ³	114	48.3	122	51.7	236	100.0

¹Tested antimicrobial: 300 mg of cephalixin sodium + 20 mg of prednisolone.

²Positive control antimicrobial: 200 mg of tetracycline + 250 mg of neomycin + 28 mg of bacitracin + 10 mg of prednisolone.

³Culture results of CM cases excluded postadmission (n = 110) are not presented in this table.

Figure 1. Least squares means and SE (bars) of overall cure outcomes of nonsevere clinical mastitis in dairy cows, according to intramammary treatment [cephapirin sodium (CS), 300 mg + 20 mg of prednisolone; or positive control (TNB): 200 mg of tetracycline + 250 mg of neomycin + 28 mg of bacitracin + 10 mg of prednisolone]. LSSCC = linear score of SCC.

sample. Therefore, 165 cases were evaluated for bacteriological cure: 85 cases in the CS group, and 81 cases in the TNB group. Least squares means for overall bacteriological cure were 0.68 (± 0.06) for CS-treated cows and 0.73 (± 0.06) for TNB-treated cows (Figure 1).

Logistic regression results showed no significant differences between treatments for overall bacteriological cure ($P = 0.54$; Table 3). On the other hand, the result of noninferiority analysis was inconclusive, as the CI crossed the pre-stated noninferiority margin (-0.15); the proportion difference was -0.067 with a 95% CI of -0.20 to 0.07 (Figure 2).

Likewise, no difference was observed between treatments when the evaluation was performed for gram-positive ($P = 0.97$) and gram-negative ($P = 0.75$) pathogen groups. For gram-positive pathogens, the overall bacteriological cure was 0.69 (90 cases out of 130). A low number of cases with gram-negative results was observed in the pretreatment culture, which could not allow us to make any statistical inferences based on the outcomes; however, overall bacteriological cure for gram-negative pathogens was 0.79 (23 out of 29; Table 4).

Pathogen Cure

A cow with a no growth or growth result in the pretreatment culture had to maintain a no growth result in both of posttreatment samples to be considered as pathogen cured. Therefore, a total of 236 cow-cases were evaluated for this outcome: 114 in the CS group and 122 in the TNB group. Logistic regression showed no difference between treatment groups ($P = 0.93$; Table 3) for pathogen cure. Least squares means for

overall pathogen cure were 0.36 (± 0.06) for CS-treated cows and 0.36 (± 0.05) for TNB-treated cows (Figure 1). Noninferiority analysis showed no risk difference of pathogen cure between the treatment groups; the proportion difference was 0.0001 with a 95% CI of -0.122 to 0.122 . The interpretation of these results is that the null hypothesis (CS was inferior to TNB) was rejected (Figure 2).

Also, treatments had no effect when the pathogen group was included in the model ($P > 0.05$). Overall pathogen cure was 0.54 for cows with no growth at pretreatment culturing (38 out of 71), 0.31 for gram-positive bacteria (41 out of 131), and 0.34 (10 out of 29) for gram-negative bacteria (Table 4).

Clinical Cure

In addition of 236 cow-cases assessed for pathogen cure, other 26 cases were added in the analysis of clinical cure. Therefore, 262 cows were included in the analysis of clinical cure: 126 in the CS group and 136 in the TNB group. However, no effect was observed in the logistic regression model comparing the treatment groups ($P = 0.08$; Table 3) in the analysis of clinical cure. Least squares means for clinical cure were 0.88 (± 0.03) for cows in the CS group and 0.94 (± 0.02) for cows in the TNB group. The result of noninferiority analysis was inconclusive (proportion difference = -0.067 ; 95% CI = -0.153 – 0.001), as the CI crossed the noninferiority margin (Figure 2).

There was no effect of treatment on clinical cure when considering gram-positive ($P = 0.31$), gram-negative ($P = 0.97$), and cultures with no growth ($P = 0.15$). Overall clinical cure was 0.91 (134 out of 147)

for gram-positive bacteria, 0.91 (29 out of 32) for gram-negative bacteria, and 0.89 (67 out of 75) for cows with no growth at pretreatment culturing (Table 5).

Extended Clinical Cure

A cow was considered extended clinical cured if she had no alterations in the milk or udder at 21 ± 3 d after CM diagnosis. Four of 236 cases assessed for pathogen cure had no data for evaluation of extended clinical cure and were excluded from this analysis; on the other hand, 18 cases were assessed for extended clinical cure, although they were not enrolled for pathogen cure (i.e., lack of culture results). Therefore, a total of 250 cow-cases were evaluated for extended clinical cure: 117 cases treated with CS, and 133 cases treated with TNB. Logistic regression showed no effect of treatment on extended clinical cure ($P = 0.83$; Table 3). Least squares means were $0.93 (\pm 0.03)$ for CS-treated cows, and $0.92 (\pm 0.03)$ for TNB-treated cows (Figure 1). Furthermore, CS treatment was noninferior for extended clinical cure in comparison to TNB treatment (proportion difference = 0.0045; 95% CI = -0.08 to 0.09 ; Figure 2).

There was no effect of treatment on extended clinical cure for gram-positive ($P = 0.93$) and gram-negative ($P = 0.41$) bacteria, and for cultures with no growth ($P = 0.33$) in the pretreatment culturing. The extended clinical cure was 0.92 (131 out of 142 cases) for gram-positive, 0.81 (26 out of 32) for gram-negative, and 0.91 (63 out of 69) for cases with no growth in the pretreatment culture (Table 5).

LSSCC Cure

Forty-five of 236 cases assessed for pathogen cure had no SCC data and were excluded from this analysis; on the other hand, 2 cases were assessed for LSSCC cure, although they were not enrolled for pathogen cure (i.e., lack of culture results). Therefore, 193 cow-cases of CM were included in the analysis of LSSCC cure: 88 in the CS group and 105 in the TNB group. Treatment had no effect on overall LSSCC reduction (≤ 4.0) posttreatment ($P = 0.62$; Table 3). Least squares means were $0.29 (\pm 0.06)$ for the CS group and $0.28 (\pm 0.06)$ for the TNB group (Figure 1). Noninferiority analysis showed no risk difference in LSSCC cure between treatment

Table 3. Mixed logistic regression model results (model 1; completed with all clinical mastitis cases, regardless of etiological group) for the evaluated cure outcomes of nonsevere clinical mastitis

Effect	n ¹	Estimate	SE	P-value	OR ²	95% CI
Bacteriological cure						
Intercept		1.58	0.57			
Treatment						
CS ³	85	-0.22	0.37	0.54	0.80	0.38–1.66
TNB ⁴	80	Reference				
Pathogen cure						
Intercept		-0.56	0.46			
Treatment						
CS ³	114	0.03	0.29	0.93	1.03	0.58–1.81
TNB ⁴	122	Reference				
Clinical cure						
Intercept		3.33	0.78			
Treatment						
CS ³	126	-0.82	0.46	0.08	0.44	0.18–1.10
TNB ⁴	136	Reference				
Extended clinical cure						
Intercept		2.43	0.81			
Treatment						
CS ³	117	0.1	0.45	0.83	1.10	0.45–2.67
TNB ⁴	133	Reference				
LSSCC cure ⁵						
Intercept		-1.47	0.53			
Treatment						
CS ³	88	0.03	0.33	0.62	1.03	0.53–2.0
TNB ⁴	105	Reference				

¹Number of cow-cases of clinical mastitis included in the analysis.

²Odds ratio.

³Tested antimicrobial: 300 mg of cephalixin sodium + 20 mg of prednisolone.

⁴Positive control antimicrobial: 200 mg of tetracycline + 250 mg of neomycin + 28 mg of bacitracin + 10 mg of prednisolone.

⁵To be considered linear score of SCC (LSSCC) cured, a cow should have SCC <200,000 cells/mL (i.e., linear score <4.0) on d 21 after enrollment; LSSCC was calculated as $LSSCC = \log_2 (SCC/100) + 3$ (Schukken et al., 2003).

Table 4. Frequency of bacteriological and pathogen cure of nonsevere clinical mastitis cases after treatment with 300 mg of cephapirin sodium + 20 mg of prednisolone (CS) or 200 mg of tetracycline + 250 mg of neomycin + 28 mg of bacitracin + 10 mg of prednisolone (TNB)

Microorganism isolated at pretreatment	Bacteriological cure		Pathogen cure	
	CS	TNB	CS	TNB
No growth	—	—	0.52 (15/29)	0.55 (23/42)
Gram-positive	0.68 (44/65)	0.71 (46/65)	0.34 (22/65)	0.29 (19/66)
CNS	0.47 (8/17)	0.62 (10/16)	0.41 (7/17)	0.31 (5/16)
<i>Staphylococcus aureus</i>	0.67 (10/15)	0.37 (3/8)	0.33 (5/15)	0.12 (1/8)
<i>Streptococcus uberis</i>	0.86 (6/7)	0.73 (11/15)	0.14 (1/7)	0.27 (4/15)
<i>Corynebacterium</i> spp.	0.80 (8/10)	0.90 (9/10)	0.20 (2/10)	0.20 (2/10)
<i>Streptococcus</i> spp.	0.33 (1/3)	0.75 (6/8)	0.33 (1/3)	0.37 (3/8)
<i>Streptococcus dysgalactiae</i>	1.00 (4/4)	0.75 (3/4)	1.00 (4/4)	0.00 (0/4)
<i>Streptococcus agalactiae</i>	0.86 (6/7)	1.00 (2/2)	0.29 (2/7)	1.00 (2/2)
<i>Bacillus</i> spp.	0.00 (0/1)	1.00 (2/2)	0.00 (0/1)	0.50 (1/2)
<i>Trueperella pyogenes</i>	1.00 (1/1)	—	0.00 (0/1)	—
<i>Nocardia</i> spp.	—	—	—	1.00 (1/1)
Gram-negative	0.71 (12/17)	0.92 (11/12)	0.35 (6/17)	0.33 (4/12)
<i>Escherichia coli</i>	0.75 (9/12)	0.89 (8/9)	0.42 (5/12)	0.11 (1/9)
<i>Serratia</i> spp.	0.67 (2/3)	1.00 (1/1)	0.00 (0/3)	1.00 (1/1)
<i>Klebsiella</i> spp.	0.00 (0/1)	1.00 (1/1)	0.00 (0/1)	1.00 (1/1)
<i>Enterobacter</i> spp.	—	1.00 (1/1)	—	1.00 (1/1)
<i>Pseudomonas</i> spp.	1.00 (1/1)	—	1.00 (1/1)	—
Other pathogen	0.67 (2/3)	1.00 (3/3)	0.00 (0/3)	0.33 (1/3)
Yeast	1.00 (2/2)	1.00 (2/2)	0.00 (0/2)	0.00 (0/2)
<i>Prototheca</i> spp.	0.00 (0/1)	1.00 (1/1)	0.00 (0/1)	1.00 (1/1)

Figure 2. Graphical representation of outcome probability differences for 300 mg of cephapirin sodium + 20 mg of prednisolone (CS) versus 200 mg of tetracycline + 250 mg of neomycin + 28 mg of bacitracin + 10 mg of prednisolone (TNB) in a noninferiority comparison. The black boxes represent the point estimates for each difference. The error bars indicate 2-sided 95% CI, and the gray shaded area indicates the zone of noninferiority. Confidence intervals crossing -0.15 (the noninferiority margin) are inconclusive. LSSCC = linear score of SCC.

groups; the proportion difference was 0.02 with a 95% CI of -0.08 to 0.09 (Figure 2).

Also, treatment had no effect on LSSCC cure for gram-positive ($P = 0.93$) and gram-negative bacteria ($P = 0.99$), or for cultures with no growth ($P = 0.33$). The LSSCC cure risk was 0.28 (32 out of 115 cases) for gram-positive pathogens, 0.29 (6 out of 21) for gram-negative pathogens, and 0.48 (26 out of 54) for cases with no growth in the pretreatment culture (Table 5).

DISCUSSION

This study was a positive-control randomized non-inferiority field trial comparing 2 commercial antimicrobials for treatment of nonsevere CM: one β -lactam, first-generation cephalosporin (cephapirin sodium; CS), and the positive control product containing a combination of antimicrobials (tetracycline + neomycin + bacitracin; TNB). Both products had prednisolone in their composition, which is a steroid anti-inflammatory compound. The noninferiority analysis showed that CS was noninferior to TNB with regard to pathogen cure, extended clinical cure, and LSSCC cure. For the primary outcomes, bacteriological and clinical cure, the noninferiority result was inconclusive due to spanning of the CI of the pre-stated noninferiority margin.

The choice of TNB as positive control in our study was mainly due to its broad-spectrum antimicrobial ac-

tivity. The TNB is an approved product marketed for intramammary treatment of mastitis in Brazil and other countries, such as France, Chile, Turkey, and Mexico. This commercial product consists of a combination of 2 antimicrobials with bactericidal activity (i.e., neomycin and bacitracin), and one with bacteriostatic activity (i.e., tetracycline). A previous study reported that combination of bactericidal and bacteriostatic drugs mostly resulted in antagonism (Ocampo et al., 2014). However, a combination of tetracycline, neomycin, and bacitracin was reported to have in vitro synergic activity against mastitis-causing bacteria (Ganière et al., 1991): neomycin + bacitracin had synergic activity against gram-positive pathogens (i.e., *Staph. aureus* and *Strep. uberis*), whereas neomycin + tetracycline had synergic activity against gram-negative bacteria (*E. coli*). The third antibiotic contained in the formulation (i.e., tetracycline for gram-positive, and bacitracin for gram-negative bacteria) did not alter the activity of other 2 antimicrobials with synergic activity (Ganière et al., 1991). The TNB was also used as control in a recent clinical trial showing high frequencies of clinical and bacteriological cures (Cortinhas et al., 2016).

A high overall risk of bacteriological cure was observed in our study (71.5%), although no difference was observed between treatment groups according to mixed logistic regression models results. The risk of bacteriological cure for CS was slightly higher in our study (68%)

Table 5. Frequency of clinical cure, extended clinical cure, and linear score of SCC (LSSCC) cure of nonsevere clinical mastitis cases after treatment with 300 mg of cephalapirin sodium + 20 mg of prednisolone (CS) or 200 mg of tetracycline + 250 mg of neomycin + 28 mg of bacitracin + 10 mg of prednisolone (TNB)

Microorganism isolated at pretreatment	Clinical cure		Extended clinical cure		LSSCC cure ¹	
	CS	TNB	CS	TNB	CS	TNB
No growth	0.83 (25/30)	0.93 (42/45)	0.96 (25/26)	0.88 (38/43)	0.45 (10/22)	0.50 (16/32)
Gram-positive	0.88 (64/73)	0.95 (70/74)	0.91 (62/68)	0.93 (69/74)	0.28 (15/53)	0.27 (17/62)
CNS	0.79 (15/19)	0.94 (16/17)	0.88 (15/17)	0.94 (16/17)	0.31 (4/13)	0.33 (5/15)
<i>Staphylococcus aureus</i>	0.88 (15/17)	1.00 (11/11)	0.94 (15/16)	1.00 (11/11)	0.31 (4/13)	0.25 (2/8)
<i>Streptococcus uberis</i>	0.86 (6/7)	0.88 (15/17)	0.86 (6/7)	0.88 (15/17)	0.33 (1/3)	0.31 (4/13)
<i>Corynebacterium</i> spp.	1.00 (11/11)	1.00 (10/10)	0.90 (9/10)	0.80 (8/10)	0.33 (3/9)	0.30 (3/10)
<i>Streptococcus</i> spp.	0.50 (2/4)	0.89 (8/9)	0.75 (3/4)	1.00 (9/9)	0.00 (0/3)	0.00 (0/8)
<i>Streptococcus dysgalactiae</i>	1.00 (5/5)	1.00 (5/5)	1.00 (4/4)	1.00 (5/5)	0.00 (0/4)	0.50 (2/4)
<i>Streptococcus agalactiae</i>	1.00 (8/8)	1.00 (2/2)	1.00 (8/8)	1.00 (2/2)	0.43 (3/7)	0.50 (1/2)
<i>Bacillus</i> spp.	1.00 (1/1)	1.00 (2/2)	1.00 (1/1)	1.00 (2/2)	0.00 (0/1)	0.00 (0/2)
<i>Trueperella pyogenes</i>	1.00 (1/1)	—	1.00 (1/1)	—	—	—
<i>Nocardia</i> spp.	—	1.00 (1/1)	—	1.00 (1/1)	—	—
Gram-negative	0.84 (16/19)	1.00 (13/13)	0.79 (15/19)	0.85 (11/13)	0.42 (5/12)	0.11 (1/9)
<i>Escherichia coli</i>	0.93 (13/14)	1.00 (10/10)	0.86 (12/14)	0.90 (9/10)	0.44 (4/9)	0.14 (1/7)
<i>Serratia</i> spp.	0.33 (1/3)	1.00 (1/1)	0.33 (1/3)	1.00 (1/1)	0.33 (1/3)	0.00 (0/1)
<i>Klebsiella</i> spp.	1.00 (1/1)	1.00 (1/1)	1.00 (1/1)	1.00 (1/1)	—	0.00 (0/1)
<i>Enterobacter</i> spp.	—	1.00 (1/1)	—	0.00 (0/1)	—	—
<i>Pseudomonas</i> spp.	1.00 (1/1)	—	1.00 (1/1)	—	—	—
Other	1.00 (4/4)	1.00 (4/4)	1.00 (4/4)	0.67 (2/3)	0.00 (0/1)	0.00 (0/2)
Yeast	1.00 (3/3)	1.00 (3/3)	1.00 (3/3)	1.00 (2/2)	0.00 (0/1)	0.00 (0/1)
<i>Prototheca</i> spp.	1.00 (1/1)	1.00 (1/1)	1.00 (1/1)	0.00 (0/1)	—	0.00 (0/1)

¹To be considered LSSCC cured, a cow should have SCC <200,000 cells/mL (i.e., linear score <4.0) on d 21 after enrollment; LSSCC was calculated as LSSCC = $\log_2$ (SCC/100) + 3 (Schukken et al., 2003).

than observed in another noninferiority trial comparing the efficacy of CS with ceftiofur hydrochloride for treatment of nonsevere CM (61%; Schukken et al., 2013). On the other hand, the bacteriological cure observed in our study was lower than that observed in the study of Apparao et al. (2009), which reported a cure risk of 84% for CM and 81% for subclinical infections. In relation to the treatment protocol using the TNB product (i.e., reference treatment), the bacteriological cure was slightly lower in our study (73%) than reported in a recent trial comparing ceftiofur hydrochloride and TNB (76%) for treatment of CM (Cortinhas et al., 2016). Differences in the frequency of bacteriological cure between studies may be attributed to factors such as microorganisms' antimicrobial susceptibility, duration of treatment, host factors (e.g., immunity status), and the concentration of the drug that can be maintained at the site of infection (Constable and Morin, 2003; Apparao et al., 2009).

Although the mixed logistic regression in our study showed no effect of treatment on bacteriological cure, the noninferiority analysis was inconclusive for this outcome. As shown in Figure 2, the CI of the risk difference for bacteriological cure crossed both the noninferiority margin and zero, which suggests that we lack the power to identify a difference between treatments for this outcome. Thus, it is not possible to make any accurate inferences about the comparison of risk differences of bacteriological cure between treatments considering the noninferiority analysis. However, based on the mixed models, we can suggest that both antimicrobials were efficient to eliminate the mastitis-causing pathogens, especially considering the high frequency of gram-positive pathogens isolated in our study. Similarly, inconclusive results were observed in another noninferiority trial comparing the risk difference of bacteriological cure between CS and ceftiofur hydrochloride for treatment of CM (Schukken et al., 2013).

One factor that can affect the sample size and potential conclusions is the choice of noninferiority margin; however, the margins that we chose were the most recommended and applied in other studies (Deluyker et al., 1999; Schukken et al., 2013; Vasquez et al., 2016). Therefore, the inconclusive result observed in our study for bacteriological cure may be attributed to the insufficient sample size. Approximately one-third of all enrolled cows were not evaluated for bacteriological cure because they were excluded postadmission. Although no difference was observed among groups regarding the frequency of postadmission losses, the cows' exclusion was more than 2 times higher than the 15% assumed in the sample size determination. In addition, a drawback of our study was not accounting for negative cultures during the sample size determination. As known, 20

to 50% of milk samples collected from CM have no microbial growth in the culture (Ruegg, 2012). In our study, 30.1% of enrolled cultures were negative, and thus, another 71 cow-cases could not be included in the analysis of bacteriological cure.

Likewise, an inconclusive result on risk differences between treatments was observed in the noninferiority analysis for clinical cure. As observed for bacteriological cure, we may have lacked the power to show difference on clinical cure between treatments, even though a greater number of cases were included in this analysis. Considering that the CI tended to lie to the right of $-\Delta$ and left to the zero (even spanning both limits; Figure 2), one could speculate that CS may have been inferior compared with TNB for clinical cure if we had a higher sample size. In addition, this speculation may be reinforced by the fact that a tendency for clinical cure ($P = 0.08$) was observed in model 1 of the logistic regression analysis. Overall, a total of 238 out of 263 cow-cases of CM (90.5%) had clinical cure 48 h after the last antimicrobial infusion, which was higher than the 62% cure risk reported elsewhere (Schukken et al., 2013). In our study, cows treated with CS had 94% clinical cure, whereas in the study of Schukken et al. (2013), both treatment protocols (i.e., cephalixin sodium and ceftiofur hydrochloride) resulted in 62% of clinical cure at approximately 10 and 17 d after the start of treatment. The higher clinical cure risk in our trial in comparison to the aforementioned study may be attributed to 2 additional (4 instead of 2) intramammary infusions of CS, and because of differences in the clinical scoring system. In addition, the commercial product containing CS in our study also had in its composition an anti-inflammatory compound (20 mg of prednisolone), which may have assisted in the reduction of clinical symptoms in comparison to the noninferiority trial conducted by Schukken and coworkers. As previously reported, anti-inflammatory compounds may be beneficial for reduction of clinical signs (Roberson, 2012).

The other primary outcome evaluated in this study was pathogen cure, and our results showed that CS was noninferior to TNB for this outcome. Although this treatment outcome is not usually reported among studies evaluating the efficacy of antimicrobials, we performed this analysis because it allowed us to also evaluate the cases with no growth in pretreatment culture. Although it is not usual to make inferences about the cure of CM cases with negative culture results, it is rational to state that regardless of the culture result a microorganism was responsible for causing the inflammatory symptoms. Considering that cows with CM are more likely to have a new case of CM within the next month after the previous case (Oliveira et al., 2015), it is reasonable to evaluate the risk of the affected quarter

to remain pathogen-free after treatment. Pathogen cure was also described in a recent randomized noninferiority trial comparing hetacillin potassium with ceftiofur hydrochloride (as reference) for the treatment of non-severe CM in dairy cows (Vasquez et al., 2016). Our overall result of 38% was lower than the 44% pathogen cure risk reported by Vasquez et al. (2016). Furthermore, the pathogen cure in our study was even lower than the 65% found by Oliveira et al. (2013), which evaluated the outcomes of treatments with any antimicrobial. The lowest pathogen cure risk in our study in comparison to other reports may be attributed to the high prevalence of *Staphylococcus aureus* in the pretreatment culture. A total of 73.9% (17 out of 23) of all *Staph. aureus* CM had isolation of any pathogen in the posttreatment cultures; and for 43.5% (10 out of 23) of cases, *Staph. aureus* was isolated in at least one of posttreatment samples, indicating the persistence of infection. In addition, the pathogen with the highest frequency of isolation in milk samples posttreatment was CNS, which was isolated in approximately 40% (57 out of 147 cases) of all cases that did not present pathogen cure. These microorganisms have been reported as minor pathogens in the mastitis epidemiology, with the potential to colonize only the canal and cistern of the teat rather than the mammary gland (Quirk et al., 2012). Therefore, some CNS isolated in milk samples collected posttreatment may have not caused an IMI.

For both secondary outcomes evaluated in our study, extended clinical cure and LSSCC cure, CS was non-inferior to TNB; also, no differences in the logistic regression models were observed. A high risk of extended clinical cure was observed for both treatments evaluated in our study (i.e., least squares means of 93% for CS-treated cows, and 92% for TNB-treated cows). For TNB-treated cows, the extended clinical cure was numerically higher than the clinical cure (i.e., evaluated approximately 48 h after the last antimicrobial infusion). This was an unexpected result, but it could be due to the low risk of pathogen cure observed in this study. Overall, the results of our study were similar to those reported by Vasquez et al. (2016) that found extended clinical cure risks >90% for both treatments (i.e., hetacillin potassium and ceftiofur hydrochloride) in evaluations performed on d 14 and 21 postenrollment.

Another secondary outcome evaluated in our study was LSSCC cure. To be considered LSSCC cured, a cow should have SCC <200,000 cells/mL (i.e., linear score <4.0) on d 21 after enrollment. The overall LSSCC cure risk in our study was 33.2%, and no difference was observed between treatments. Our result was greater than overall LSSCC cure risk (22%) found in another study evaluating the efficacy of standard versus ex-

tended intramammary cefquinome treatment of CM in cows with persistent high SCC (Swinkels et al., 2014). The higher SCC recovery observed in our study in comparison to the aforementioned report may be attributed to the fact that the cases included in our study were clinical rather than persistent subclinical cases. In addition, our study evaluated only milk samples collected on d 21, whereas Swinkels and coworkers collected 2 milk samples for SCC (i.e., d 14 and 21). On the other hand, the LSSCC cure of our study was lower than that observed in a report characterizing short-term posttreatment outcomes after CM treatment in a single quarter using ceftiofur hydrochloride (58.2%; Pinzon-Sanchez and Ruegg, 2011). Differences between studies on success of IMI elimination and SCC recovery after treatment may also be due to cow factors such as mastitis-causing pathogen, SCC and number of CM cases before the current infection, age and stage of lactations at the mastitis occurrence, and posttreatment interval for milk sample collection (Sol et al., 2000; Bradley and Green, 2009; Pinzon-Sanchez and Ruegg, 2011).

In our study, we also compared the efficacy of CS and TNB on cure risk of specific pathogen groups (gram-negative, gram-positive, and no growth), but no effect of treatments was found for any of evaluated outcomes. Other reports also did not find difference between treatments when the etiological groups were evaluated by logistic regression models (Cortinhas et al., 2016; Vasquez et al., 2016). In the study of Vasquez et al. (2016), noninferiority analysis was completed for gram-positive and gram-negative etiological groups, and the results were inconclusive potentially because of the small sample size of pathogen groups. On the other hand, Schukken et al. (2013) concluded that farm-specific treatment protocols that differ for gram-positive and gram-negative may be developed, as ceftiofur showed a significantly higher bacteriological cure of gram-negative cases in comparison to CS.

First-generation cephalosporins were described to have antimicrobial activity against gram-positive bacteria, but only limited gram-negative activity (Guérin-Fauble et al., 2003; Pfeifer et al., 2010). In our study, because of the low number of gram-negative pathogens in pretreatment culture, we could not make strong statistical inferences on the effect of treatment on cure outcomes of this pathogen group. Therefore, it can be argued that a larger number of gram-negative cases could favor TNB compared with CS on cure risks of IMI caused by this group of pathogens.

Despite the large number of milk samples submitted for microbiological examination in our study (i.e., milk samples collected pre- and posttreatment), we found no mixed-culture results. This was an unexpected outcome, especially because a milk sample was defined as culture

positive by the presence of a single microorganism of a particular morphology. Although most studies have reported a frequency of mixed culture ranging from 3.0 to 6.5% of total cultures (Olde Riekerink et al., 2008; Oliveira et al., 2013; Verbeke et al., 2014), other studies also did not report mixed cultures in milk samples from cows with CM (Schukken et al., 2013; Vasquez et al., 2016).

One limitation of our study was that collected data did not allow performing a noninferiority analysis according to pathogen group. In sample size calculation, we did not plan for stratification of data based on culture results, which prevented us from making accurate inferences on the outcomes when the cases were stratified by etiology. Additionally, we had a higher loss to follow-up than expected in the sample size determination, especially because of occurrence of mastitis in another quarter after enrollment. For example, after postadmission exclusion, only 39 gram-negative cases were evaluated in the data analysis. Therefore, it was only possible to perform the logistic regression analysis according to pathogen group. A study with higher sample size could result in different outcomes, especially for CM caused by gram-negative pathogens. The evaluation of treatment efficacy on CM cure risks stratified by etiology was not the primary objective of this study. However, the comparison of group frequencies and descriptive results contributed for a better understanding of overall outcomes (regardless of culture results).

Another limitation was the nonaccomplishment of blinding of farm personnel to treatments, although both the farm and laboratory personnel were unaware of the treatment outcomes in our study. One could say that the farm personnel might have been familiar with the commercial antimicrobials used, which could have introduced ascertainment bias in the study. However, because no statistical differences were observed among groups during randomized allocation of cows to treatments, we considered that ascertainment bias was avoided.

Despite the limitations, both commercial antimicrobials evaluated in our study showed a substantial overall risk of bacteriological and clinical cure. These results may be in the interest of pharmaco-epidemiology, especially considering the current global concern about the prudent use of antimicrobials in livestock (Van Boeckel et al., 2017). In addition to the efficiency of evaluated antimicrobials on CM cure risks, none of the active ingredients are currently classified by the World Health Organization as critically important for human health. Instead, they are composed of active ingredients commonly used in veterinary medicine, which have been described previously as poorly effective against mastitis-causing pathogens *in vitro* (e.g., tetracycline;

Rajala-Schultz et al., 2004; Rato et al., 2013), or have narrow-spectrum activity (i.e., cephalixin sodium). Therefore, these results may help veterinary practitioners and farm personnel in the day-to-day decision-making regarding CM therapy.

CONCLUSIONS

Our study compared whether a treatment protocol using CS was noninferior to TNB by the evaluation of differences in the cure risks. We conclude that CS was noninferior compared with TNB in regard to overall pathogen cure, extended clinical cure, and LSSCC cure. Inconclusive results in the noninferiority analysis were observed for clinical and bacteriological cure. No differences were observed between CS and TNB on the cure outcomes when the data were stratified by etiology groups (gram-negative, gram-positive, and cultures with no growth).

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