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Ceftiofur efficacy in the intramammary extended treatment of staphylococcal subclinical mastitis in primiparous cows

Eficácia do ceftiofur no tratamento estendido intramamário da mastite subclínica por estafilococos em vacas primíparas

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Highlights

Effectiveness of intramammary ceftiofur in extended treatment in cases of mastitis.
Treated groups (G3 + G4) and untreated (G2), cure rates were 60% and 26.7%.
Extended intramammary therapy with ceftiofur in bovine primiparous cows is effective.

Abstract

Extended therapy is an alternative approach to treat nonresponsive staphylococcal mastitis, although it has been used mainly in adult cows. This study investigated the efficacy of intramammary ceftiofur in the treatment of staphylococcal subclinical mastitis in primiparous cows. Sixty primiparous cows Holstein were divided into four groups (G1 = Group negative to staphylococci infection, G2 = Group positive to staphylococci infection untreated with intramammary ceftiofur, Group, G3 = Group treated with intramammary ceftiofur early lactation, G4 = Group treated with intramammary ceftiofur late lactation group) with milk samples collected at five moments (M0 = diagnosis of subclinical mastitis, M1 = 7 days after diagnosis of subclinical mastitis and early extended therapy, M2 = 14 after the diagnosis of subclinical mastitis, M3 = 21 days after diagnosis of mastitis) and M4 = 28 days after diagnosis of mastitis). In groups G3 and G4, which were treated with intramammary ceftiofur, the microbiological cure was evidenced by a reduction in SCC of 73.3% and 46.7%, respectively. Thus, in the treated groups (G3 + G4) and untreated (G2), cure rates were 60% and 26.7%, respectively (P <0.05). Forty-five strains of staphylococci were

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isolated, with a predominance of S. aureus (51,1%) and S. intermedius (48,8%). The in vitro antimicrobial susceptibility pattern showed higher sensitivity indices of isolates for oxacillin (80%), ceftiofur (77.8%), cephalexin (77.8%), ciprofloxacin (66.7%), and gentamicin (60%). In contrast, cloxacillin (24.4%), penicillin (35.6%), and tetracycline (44.5%) were ineffective antimicrobials for staphylococci isolates. Among the milk samples taken when M0 and M1 (7 days after isolation), 13.3% were positive for the detection of the inhibiting substances (antimicrobials) in Delvotest™ and all negative in the Snap test™, whereas in the M2 (14 days after isolation), 55% were positive in Delvotest™ and 46.7% in the Snap test™. Twenty-one days after diagnosis (M3), 16.7% revealed positive results in Delvotest™ and 11.7% in the Snap test™. For M4 (28 days after isolation), 11.7% showed positive results in Delvotest™, and 8.3% revealed positive results in the Snap test™. There was a reduction in SCC among all cured animals. Significant results were identified for milk production in the M0, so G1 produced more milk than the other three groups, G2, G3, and G4. A statistical association was observed between the reduction in the volume of daily milk and production in animals with subclinical mastitis, denoting losses in milk production in primiparous bovines infected during lactation by staphylococci. Extended intramammary therapy with ceftiofur in bovine primiparous cows subclinically affected by staphylococcal mastitis is effective mainly in cases treated at the beginning of lactation, showing microbiological cure and reduction in SCC.
Key words: Cefalosporin. *Staphylococcus aureus.* Mammary. Mastitis. Cows. Extended therapy.

Resumo

O estudo presente investigou a eficácia do ceftiofur intramamário no tratamento da mastite subclínica por estafilococos em primíparas bovinas. Foram utilizados 60 animais da raça holandesa, divididos em quadro grupos (G1 = Grupo negativo, G2 = Grupo positivo não tratado com ceftiofur intramamário, G3 = Grupo tratado com ceftiofur intramamário no início da lactação, G4 = Grupo tratado com ceftiofur intramamário no final da lactação), com amostras de leite coletadas em cinco momentos (M0 = diagnóstico da mastite subclínica, M1 = 7 dias após diagnóstico e início da terapia estendida, M2 = dia 14 após o diagnóstico da mastite subclínica, M3 = dia 21 após o diagnóstico da mastite, M4 = dia 28 após o diagnóstico da mastite). Nos grupos tratados com ceftiofur intramamário, foi evidenciada a cura microbiológica com redução na CCS em 73,3% no G3 e 46,7% no G4. Nos grupos tratados (G3+G4) e não tratado (G2), as taxas de cura foram, respectivamente, 60% e 26,7% ($p<0,05$). Foram isoladas 45 linhagens de estafilococos, com predomínio de S. aureus (51,1%) e S. intermedius (48,8%). O perfil de sensibilidade microbiana "in vitro" dos isolados apresentou maiores índices de sensibilidade para oxacilina (80%), ceftiofur (77,8%) e cefalexina (77,8%), ciprofloxacino (66,7%) e gentamicina (60%). Em contraste, cloxacilina (24,4%), penicilina (35,6%) e tetraciclina (44,5%) foram os antimicrobianos menos efetivos diante dos estafilococos isolados. Nas amostras de leite colhidas nos momentos M0 e M1, 13,3% foram positivas para o teste de detecção de substâncias inibidoras na prova de Delvotest™ e todas negativas no Snap test™. Já, no M2, 55% foram positivas no Delvotest™ e 46,7% no Snap test™. No M3, 16,7% positivas no Delvotest™ e no 11,7% no Snap test™. No M4, 11,7% positivas no Delvotest™ e 8,3% no Snaptest™, dois dias após o término do tratamento (M2). Foram identificados nos grupos G3 e G4 a presença de animais positivos em 96,7% para o Delvotest™ e 90% para o Snap test™. Ocorreu redução na CCS em todos os animais curados. Foram identificados resultados significativos para a produção de leite no MO, posto que o G1 produziu mais leite

se comparado aos outros três grupos G2, G3 e G4. Foi observada associação estatística entre a redução no volume de leite diário e a produção nos animais com mastite subclínica, denotando prejuízos na produção de leite em primíparas bovinas infectadas na lactação por estafilococos. A terapia intramamária estendida com ceftiofur em primíparas com mastite estafilocócica é eficaz principalmente nos casos tratados no início da lactação, mostrando cura microbiológica e redução da CCS, indicando que esta abordagem terapêutica alternativa pode ser utilizada para o tratamento de novilhas.
Palavras-chave: Cefalosporinas. *Staphylococcus aureus.* Mama. Mastite. Vaca. Terapia estendida.

Introduction

Mastitis is the major disease that affects the dairy industry, including heifers. It is characterized by the highest occurrence of subclinical cases in the first postpartum days (Nitz et al., 2020). Coagulase-positive staphylococci (CPS) cause long-term infections, with a tendency toward chronicity, high cellularity, low cure rates, and great loss in milk production (Swinkels et al., 2013).

The difficulty in treating staphylococcal mastitis, particularly *S. aureus,* due to the high occurrence of this group of agents in bovine mastitis, the multidrug resistance to conventional antimicrobials, a set of virulence factors, and mechanisms of evasion of the pathogen's immune system have motivated programs for the early disposal of bovine females with mastitis by *S. aureus,* which cause a negative impact on herds (Campos et al., 2022).

In the last decade, extended therapy has emerged as an alternative treatment, particularly for herds with a high prevalence of subclinical staphylococcal mastitis refractory to conventional therapy, caused mainly S. aureus (Truchetti et al., 2014; McDougall et al., 2019). The extended therapy consists of 6 to 10 applications of antimastitics via the intramammary route to increase the rates of clinical and bacteriological cure, as well as to reducing the somatic cell count (SCC) and decrease the quality and daily milk production of the animals (Oliver et al., 2004; Kasravi et al., 2011; Swinkels et al., 2013).

To date, the studies conducted on investigate the extended therapy have no focused on the lack of monitoring somatic cell count, or even milk production before and after treatment (Roy et al., 2009; Swinkels et al., 2013; McDougall et al., 2019). In addition, most of studies of extended therapy have been perfomed in adult dairy cows.

Considering the virulence of staphylococci-induced infections in dairy cows, the low therapeutic response of conventional treatments during lactation in staphylococcal bovine mastitis, and the current tendency to culling cows with chronic staphylococcal mastitis, the present study investigated the effectiveness of intramammary ceftiofur in extended treatment in cases of subclinical staphylococcal mastitis in naturally infected primiparous cows, where bacteriological cure, somatic cell count (SCC), and the presence of pharmacological (antimicrobial) residues were assessed 28 days after extended treatment.

Materials and Methods

Study design

Bacteriological cure, somatic cell count (SCC), and the presence of antimicrobial residues after extended intramammary treatment were investigated in bovine primiparous cows naturally infected by subclinical mastitis using ceftiofur.

Animals and farms

Sixty primiparous, Holstein breeders, aged 3 to 4 years, from 15 different dairy farms were used. Farms and primiparous cows were eligible for sampling if they met the following criteria: 1) lactation period between 15 and 200 days, 2) herds with a daily average between 15 to 17 liters/day, 3) diagnosis of subclinical staphylococcal mastitis, 4) animals without previous treatment during lactation with other antimicrobials, and 5) farms with mechanical milking. A single animal was collected from each property for each experimental group. The primiparous cows belonged to farms from Paraná state from Brazil. The climate of farms sampled is characterized by tropical temperatures. The calves were from medium-scale farms (defined here as farm holdings between 20-200 hectares), with a different average size of herds. All the animals were breeding in the pasture, with similar conditions of nutrition, management facilities, technical degree, and sanitary conditions, including mandatory vaccines.

Diagnosis of subclinical and clinical mastitis

Subclinical and clinical mastitis were diagnosed by the California Mastitis Test (CMT) and strip cup test. The mammary quarter of primiparous cows that showed a positive reaction 2+ or 3+ scores in CMT were submitted to milk collection for microbiological diagnosis.

Milk samples

Milk samples obtained exclusively from primiparous cows with subclinical mastitis were collected after antisepsis of the teats with iodine-povidone solution (2%), drying with disposable paper towels, and elimination of the first jets of milk, followed by antisepsis of the galactophore channel with 70% alcoholl. The milk (5 mL) was collected in sterile tubes with a screw cap, immediately refrigerated (4-8 Co), and sent to microbiological culture.

Treatment groups

Four aleatory groups were formed (G1, G2, G3, and G4) to evaluate the extended treatment of subclinical mastitis as follows:

G1 (negative group): 15 primiparous cows with negative CMT, negative microbiological isolation, and SCC <200,000 cells/mL;

G2 (untreated positive group): 15 primiparous cows with positive CMT (2+ or 3+), positive isolation of staphylococci and SCC> 200,000 cells/mL, not treated with ceftiofur by intramammary route;

G3 (positive group treated at the beginning of lactation): 15 primiparous cows with positive CMT (2+ or 3+), positive isolation of staphylococci and SCC> 200,000 cells/mL, with lactation between 15 to 100 days, submitted to extended intramammary treatment with ceftiofur for five days with application every 12 hours;

G4 (positive group treated in the middle of lactation): 15 primiparous cows with positive CMT (2+ or 3+), positive isolation of staphylococci and SCC> 200,000 cells/mL, with lactation between 101 to 200 days, submitted to extended intramammary treatment with ceftiofur for five days, applied every 12 hours.

Only a mammary quarter per animal was considered for the constitution of the groups, making 60 animals for the formation of the four groups.

Extended intramammary treatment

Intramammary treatment was performed according to proper antisepsis conditions. It was instituted 7 days after the isolation of staphylococci in the milk samples of primiparous cows with subclinical mastitis, after previous *in vitro* susceptibility pattern of the staphylococcal isolates to ceftiofur. The animals from groups G3 and G4 received intramammary treatment every 12 hours for five consecutive days, totaling ten applications of antimastitic based on ceftiofur (Spectramast™ - 125 mg/10 mL - Pfizer Animal Health, New York, EUA).

The study was divided into five moments (M) called MO, M1, M2, M3, and M4, namely:

MO or zero-day = diagnosis of subclinical mastitis (CMT), isolation of staphylococci, and *in vitro* susceptibility test of the isolates to ceftiofur;

M1 = 7 days after the diagnosis of subclinical mastitis (CMT) and the start of extended therapy;

M2 = 2 days after the end of the extended therapy (corresponding to the 14th day after the diagnosis of subclinical mastitis);

M3 = 7 days after M2 (corresponding to day 21 after mastitis diagnosis or 9 days after the end of treatment);

M4 = 7 days after M3 (corresponding to day 28 after mastitis diagnosis or 16 days after the end of treatment).

Isolation and phenotypic characterization of staphylococci

Milk samples from animals with subclinical mastitis were collected aseptically from all animals in the four groups throughout the study (M0, M1, M2, M3, and M4). Then, they were plated on bovine blood agar (5%) and MacConkey agar incubated at 37°C under aerobic conditions for 72 hours. The speciation of microorganisms was performed according to conventional phenotypic methods (Quinn et al., 2011).

In vitro antimicrobial susceptibility profile

All staphylococci isolated from animals with subclinical mastitis in the positive groups (G2, G3, and G4) were submitted to the *in vitro* antimicrobial susceptibility test based on the disc diffusion method (Clinical and Laboratory Standards Institute [CLSI], 2020)

using nine antimicrobials from four classes, as follows: aminoglycosides (gentamicin 10 µg), beta-lactams and derivatives (cephalexin 25 µg, ceftiofur hydrochloride 30 µg, cloxacillin 25 µg, oxacillin 1 µg, penicillin 40 µg, sodium cefoperazone 75 µg), fluoroquinolones (ciprofloxacin 5 µg), and tetracyclines (tetracycline 15 µg). Isolates that exhibited simultaneous resistance to >3 different classes (groups) of antimicrobials were considered multiresistant (Magiorakos et al., 2012). Intermediate results of in vitro antimicrobial pattern were not included in analysis of multiresistant isolates.

Electronic somatic cell count

All milk samples from sampled animals at different times of the study (MO, M1, M2, M3, and M4) were placed in appropriate plastic tubes and homogenized with cellular milk preservative (bronopol), aiming at SCC in electronic equipment (Somacount 500, Bentley Instruments, Minnesota, EUA).

Antimicrobial residues (antimicrobials) in milk

The presence of antimicrobial residues was investigated in all milk samples (M0, M1, M2, M3, M4) using a commercial kit (Delvotest™, DSM Food Specialties Dairy Ingredients, Netherlands). Simultaneously, the same samples were also submitted to another commercial kit (Snap-test™, DSM Food Specialties Dairy Ingredients, Netherlands), aiming at the detection of antimicrobial residues belonging to the beta-lactam class, according to the manufacturer's recommendations.

Monitoring of animals

Over the 28 days of the study from the diagnosis of subclinical infection, the animals were monitored for physiological parameters as well as examined of the mammary gland, and performed the CMT and strip cup tests (Constable et al., 2016).

Evaluation of bacteriological cure

The bacteriological cure was considered in the mammary quarters that did not show the isolation of the same staphylococci species identified in M0, M1, M3, and M4.

Exclusion criterion

During intramammary treatment of groups, were excluded the animals that present the following conditions: (1) developed clinical and/or systemic signs of mastitis, (2) *Staphylococcus* isolate was not susceptible to ceftiofur after *in vitro* test, (3) animals affected by other infections, (4) impossibility of treatment at any time of the study, (5) impossibility of collect material at any time of the study, and (6) presence of antimicrobial residues in the commercial tests at moments M0 and M1. Thus, 880 primiparous cows with a diagnosis of subclinical mastitis were required to establish the studied groups.

Statistical analyses

The different moments (M0, M1, M2, M3, M4) were compared according to the effectiveness of bacterial cure of ceftiofur

using in the extended treatment and the reduction in SCC based on the Chi-square test, with a level of significance P <0.05.

Preliminarily, the continuous and ordinal variables were submitted to the Shapiro-Wilk test to prove the assumption of normality of the data, which found that only the production of milk in kg/cow/day presented a distribution parametric. To determine if average milk production differed between groups, the Levene test was initially used to validate the homogeneity of variances. Once the assumption was rejected, analysis of variance was used. in one-way (one-way ANOVA) with Welch correction and contrasts by the Games-Howell.

To determine whether the averages of milk production differed between moments, within each group, the analysis of variance test was used for repeated measures, with validation of the assumption of sphericity of data by the method of Mauchly and contrasts by the Sidak method. In cases where the assumption of data sphericity was violated, the Greenhouse-Geisser correction was applied. To determine whether somatic cell counts and CMT scores differed between groups, the nonparametric Kruskal-Wallis test was used with the contrast Student-Newman-Keuls method. To compare these parameters between moments, within each group, Friedman's nonparametric test was used, with contrasts by Dunn's method.

All comparisons were performed in SPSS software (version 16.0), and Biostat 5.3, considering a 5% significance level (Field, 2012). The sample size calculation was according to Daniel (2009), in which it was determined by a mathematical model that a minimum of 24 milk samples from primiparous cows were affected by subclinical staphylococcal mastitis at the study. The results obtained were analyzed using general descriptive statistics in the BioStat™ statistical program (5.3 version), considering a 95% confidence interval and significance level of P <0.05.

Results and Discussion

Microbiological cure

The results of the microbiological cure are summarized in Table 1. Microbiological cure with reduction in spontaneous SCC for subclinical mastitis caused by staphylococci could be seen in three animals (20%). Only in one animal (6.7%) revealed a microbiological cure without a reduction in SCC in the group (G2), which did not receive any antimicrobial treatment during the study. In groups undergoing intramammary treatment with ceftiofur hydrochloride, cure was observed microbiological analysis with reduction in SCC in 11 animals (73.3%) in the group treated at the beginning of lactation (G3), and in seven animals (46.7%) in the group treated at the end of lactation (G4).

Table 1
Microbiological cure and reduction in somatic cell count after extended intramammary therapy with ceftiofur in primiparous cows with subclinical mastitis naturally infected by coagulase-positive staphylococci

Study Groups	Microbiological cure and reduction in somatic cell count	Microbiological cure without a reduction in somatic cell count	Uncured animals
	no animals/ total group (%)	no animals/ total group (%)	noanimals/ total group (%)
G1	-	-	-
G2	3/15 (20%)[a]	1/15 (6,7%)	11/15 (73.3%)[a]
G3	11/15 (73.3%)[b]	-	4/15 (26.7%)[b]
G4	7/15 (46.7%)[c]	-	8/15 (53.34%)[c]

p = Values of significance for comparison between groups by the analysis of variance test, in the same column, followed by different lowercase letters differ statistically. G1 = Negative group, G2 = Positive group not treated, G3 = Group treated at the beginning of lactation, G4 = Group treated at the end of lactation.

In the treated (G3+G4) and untreated (G2) groups, the microbiological cure rates were 60% (n= 18/30) and 26.7% (n= 4/15), respectively, showing significant results (P<0.05) between these variables. *In vitro* unsucessful of ceftiofur was observed in 26.7% (n= 4/15) for the group treated at the beginning of lactation (G3) and 53.3% (n= 8/15) in the group treated in the middle of lactation (G4), even with the treatment being supported by the in vitro sensitivity pattern of the isolates.

Microbiological culture

Forty-five staphylococci strains were isolated in groups G2, G3, and G4, including Staphylococcus aureus (51.1%/23) and Staphylococcus intermedius (48.9%/22).

In vitro antimicrobial susceptibility pattern

The staphylococcal isolates showed greater susceptibility to oxacillin (80%), ceftiofur (77.8%), cephalexin (77.8%), ciprofloxacin (66.7%), cefoperazone (60%), and gentamicin (60%). In contrast, cloxacillin (24.4%), penicillin (35.6%), and tetracycline (44.5%) were the least effective antimicrobials against the staphylococci isolates.

The isolates were less resistant to antimicrobials belonging to the cephalosporin class. Particularly for ceftiofur, only 11.1% of partially sensitive and 6.6% resistant isolates were identified. Multidrug resistance isolates were observed in 5/15 (33.3%), 3/15 (20%), and 3/15 (20%) staphylococcal strains isolated in G2, G3, and G4, respectively, which corresponds to 28.9% (n = 13/45) of all studied isolates. It was also possible to observe that no single bacterial strain was susceptible to all tested antimicrobials, even though the study was conducted on primiparous cows.

Presence of antimicrobial residues

Among the 60 milk samples collected at M0, all samples were negative for Snap test™. This result was observed also in M1 (Table 2). The simultaneous use of two commercial techniques to identify antimicrobial residues revealed that ceftiofur, when administered through the intramammary route, still presents residues in most animals two days after the end of treatment (M2), since in G3 and G4, the presence of positive animals was 96.7% and 90% for commercial Delvotest™ and Snap test™, respectively.

Table 2
Identification of antimicrobial residues and/or substances that inhibit bacterial growth - using two commercial tests - in the milk of primiparous cows with and without subclinical staphylococcal mastitis, according to different moments after the extended intramammary therapy with ceftiofur

	G1*		G2		G3		G4		Positive Total	
	Delvo* test™	Snap** test™	Delvo test™	Snap test™	Delvo test™	Snap test™	Delvo test™	Snap test™	Delvo test™	Snap test™
M0**	13.3% (2/15)	- (0/15)	13.3% (2/15)	- (0/15)	13.3% (2/15)	- (0/15)	13.3% (2/15)	- (0/15)	13.3% (8/60)	- (0/60)
M1	13.3% (2/15)	- (0/15)	13.3% (2/15)	- (0/15)	13.3% (2/15)	- (0/15)	13.3% (2/15)	- (0/15)	13.3 (8/60)	- (0/60)
M2	13.3% (2/15)	- (0/15)	13.3% (2/15)	6.66 (1/15)	93.3% (14/15)	93.3% (14/15)	100% (15/15)	86.7% (13/15)	55% (33/60)	46.7% 28/60
M3	13.3% (2/15)	6.7% (1/15)	20% (3/15)	6.66% (1/15)	20% (3/15)	13.3% (2/15)	13.3% (2/15)	20% (3/15)	16.7% (10/60)	11.7% (7/60)
M4	13.3% (2/15)	6.7% (1/15)	20% (3/15)	13.33% (2/15)	6.7% (1/15)	13.3% (2/15)	6.7% (1/15)	6.7% (1/15)	11.7% (7/60)	8.3% (5/60)

* G1 = negative group, G2 = positive group not treated, G3 = group treated at the beginning of lactation, G4 = treated group at the end of lactation, ** M0 or day zero = diagnosis of subclinical mastitis and isolation of staphylococci, M1 = 7 days after diagnosis of subclinical mastitis and initiation of expanded therapy, M2 = 2 days after the end of extended treatment (corresponding to day 14 after diagnosis of subclinical mastitis), M3 = 7 days after M2 (corresponding to day 21 after diagnosis of mastitis or 9 days after the end of treatment), M4 = 7 days after M3 (corresponding to day 28 after the diagnosis of mastitis or 16 days after the end of treatment).
*Delvotest™= DSM Food Specialties Dairy Ingredients, Netherlands.
**Snap test™= DSM Food Specialties Dairy Ingredients, Netherlands.

In M3 corresponding to day 21 after mastitis diagnosis or 9 days after the end of treatment, 16.7% of the animals showed positive results in both tests. At M4 (16 days after the end of treatment), 6.7% of the animals were still positive for Delvotest™, and 10% were positive for Snap test™, considering only the 30 animals that underwent intramammary treatment.

Somatic cell count (SCC) and CMT scores

A statistically significant association ($p<0.001$) was observed for the SCC medians between group G1 (negative) and G2, G3, and G4 (positive for staphylococci) groups, since G1 showed normal values of cellularity (66x10³ cells/mL), while animals of G2 (1.987x10³ cells/mL), G3 (1.320x10³ cells/mL) and G4 (1.234x10³ cells/mL), with staphylococcal mammary infection, showed higher number of cells than the reference values of healthy cows. The groups G3 and G4 showed a significant reduction of the cell a decrease of the CMT scores. Similarly, the untreated group (G2) also showed a reduced cell number and CMT scores in animals that showed spontaneous cure in Table 3

Table 3
Relationship between CCS in bovine primiparous cows and the CMT reaction, spontaneous microbiological cure, and/or response in extended intramammary therapy with ceftiofur

Animals	G2 SCC x 10³ cels/mL		G2 Score CMT		G3 SCC x 10³ cels/mL		G3 Score CMT		G4 SCC x 10³ cels/mL		G4 Score CMT	
	M0	M4	M0	M4	M0	M4	M0	M4	M0	M4	M0	M4
1	2345	987	3+	3+	1666	543	3+	2+	4312	888	3+	3+
2	1765	765	3+	2+	5210	9873	3+	3+	900	421	2+	2+
3	1654	655	3+	2+	1111	342	3+	1+	1345	521	3+	2+
4	899	543	2+	2+	2981	1010	3+	3+	1341	277	3+	1+
5	-	-	-	-	1234	301	3+	1+	987	211	2+	1+
6	-	-	-	-	777	265	2+	1+	787	376	2+	1+
7	-	-	-	-	1890	287	3+	1+	698	555	2+	2+
8	-	-	-	-	2109	543	3+	2+	-	-	-	-
9	-	-	-	-	2870	657	3+	1+	-	-	-	-
10	-	-	-	-	909	241	2+	2+	-	-	-	-
11	-	-	-	-	1008	241	3+	2+	-	-	-	-
Median SCC x 10³ cells/mL (exclusive for cured animals	1709[a]	710[b]			1666[a]	417[c]			987[b]	398[c]		

SCC = somatic cell count, cells \ mL = cells \ milliliter, reference value 200,103 cells \ mL CCS for healthy cows. G1 = negative group, G2 = positive group not treated, G3 = group treated at the beginning of lactation, G4 = group treated at the end of lactation, M0 or zero-day = diagnosis of subclinical mastitis and isolation of staphylococci, M4 = 7 days after M3 (corresponding to day 28 after the diagnosis of mastitis or 16 days after the end of treatment). Median CCSx103 cells/mL exclusively for animals that obtained spontaneous microbiological cure in the group (G2) and/or cure after extended therapy in groups G3 and G4. p = Values of significance for comparison between groups by the test of analysis of variance in one way the same column, followed by different lowercase letters differ statistically.

Milk production

The data referring to milk production (kg/animal/day) are grouped in Table 4.

Table 4
Values of milk production in liters/animals expressed as the mean ± standard deviation in four experimental groups evaluated at five different times in primiparous cows, with and without staphylococcal mastitis after extended intrammary therapy with ceftiofur

Groups	Moments					
	M0	M1	M2	M3	M4	P*
G1	15.6 ± 0.6 (Aa)	16.3 ± 0.7 (Ab)	15.6 ± 0.6 (Aa)	15.8 ± 0.7 (Aac)	14.8 ± 0.9 (Aa)	<0,001
G2	14.4 ± 1.6 (Aba)	14.5 ± 1.8 (Ba)	13.3 ± 1.7 (Bbc)	12.7 ± 1.9 (Bc)	12.1 ± 2.1 (Bc)	<0,001
G3	13.4 ± 1.5 (Ba)	15.7 ± 0.4 (Bb)	13.3 ± 1.7 (Ba)	14.2 ± 1.8 (Bab)	14.5 ± 2.1 (Aab)	<0,001
G4	13.7± 1.2 (Ba)	15.5 ± 0.8 (Bb)	12.8 ± 1.2 (Ba)	13.2 ± 1.3 (Ba)	13.1 ± 2.2 (Aba)	<0,001
P**	< 0.001	0.001	< 0.001	< 0.001	0.001	-

p* = Significance values for comparison between moments using the analysis of variance test for repeated measures; p ** = Significance values for comparison between groups by one-way analysis of variance test; Averages in the same column, followed by different capital letters, differ statistically; Averages on the same line, followed by different lowercase letters, differ statistically. M0 or day zero = diagnosis of subclinical mastitis and isolation of staphylococci, M1 = 7 days after diagnosis of subclinical mastitis and initiation of expanded therapy, M2 = 2 days after the end of extended treatment (corresponding to day 14 after diagnosis of subclinical mastitis), M3 = 7 days after M2 (corresponding to day 21 after mastitis diagnosis or 9 days after the end of treatment), M4 = 7 days after M3 (corresponding to day 28 after mastitis diagnosis or 16 days after the end of treatment).

In this study, extended intramammary therapy with ceftiofur in bovine primiparous cows infected by staphylococcal species with subclinical masttis was effective mainly in cases treated at the beginning of lactation, showing microbiological cure and reduction in SCC, besides a persistence of antimicrobials residues in milk of treated animals.

Microbiological cure rates for bovine staphylococcal mastitis have ranged between 13 and 74% of cases, depending on several factors, including the active principle and route of administration, lactation period or dry cow, and treatment modality or type (McDougall et al., 2022). Intramammary extended therapy during lactation of dairy cattle, using 6 to 10 antimicrobial applications, has been proposed as a alternative treatment approaches for multidrug resistant bacteria (McDougall et al., 2019), particularly indicated for cases of chronic subclinical mastitis-related *S. aureus* (Roy et al., 2009) in animals of high economic values.

A recent study observed that the duration of intramammary treatment, as well as the lactation phase of the cow (beginning, middle, or end), are decisive factors in the microbiological cure rates of subclinical

staphylococcal mastitis in cows and heifers (McDougall et al., 2022). The option for intramammary extended therapy in heifers, at the beginning of lactation, has recently been recommended when the treatment is supported by *in vitro* susceptibility tests, especially in mastitis caused by *S. aureus*, since it has enabled the recovery of the production of daily milk, reduction in SCC and significant cure in lactation (McDougall et al., 2019). In fact, similar results were obtained in the present study, since the microbiological cure rate of subclinical mastitis in the animals sampled at the beginning of lactation was 73.4%, and reduction of SCC after 5 days of treatment. These results agree with those obtained by Middleton (2005), who obtained an 80% cure rate in subclinical staphylococcal mastitis on the fourth day after extended treatment with third-generation cephalosporins, although relapse was reported among 30% of cases after 28 days of end of therapy.

In the present study, significant differences in efficacy were identified between all treatment regimens using ceftiofur (G3 and G4) when compared to the positive untreated group (G2). In the statistical analysis, when the group treated at the beginning of lactation (G3) was compared to the group treated at half lactation (G4), it was observed that the two types of treatment were different, showing a statistical significance in the bacterial cure that was higher in primiparous 15 to 100 days postpartum (G3), as opposed to animals with lactation between 101 and 200 days (G4). There was also a 26.7% spontaneous cure among animals sampled. This result agree with reveals by Bradley and Green (2009) and Sol et al. (2000), who obtained spontaneous cure rates in heifers of 31.9% and 38.8%, respectively.

The duration of intramammary treatment and the lactation phase of the cow (beginning, middle or end), are factors that influence the microbiological cure rates of subclinical staphylococcal mastitis in cows and heifers (Roy et al., 2009). The option for extended intramammary therapy in heifers of high economic value, at the beginning of lactation, has recently been recommended when the treatment is supported by *in vitro* susceptibility test especially in mastitis associated-*S. aureus,* as it has enabled the recovery of daily milk production, a reduction in SCC, and a significant cure in lactation (Nitz et al., 2020).

These results indicate that, similar to the conventional treatment in the course of lactation, the highest cure rates are obtained in animals at the beginning of lactation, since the recurrence of cases (in the same lactation) is apparently due to the chronicity of the infection, related to the formation of microabscesses in the parenchyma and intracellular maintenance of the agent, which makes the therapeutic resolution of cases difficult (Langoni et al., 2017).

Few studies have evaluated the extended treatment of subclinical mastitis by staphylococci in primiparous cows (Roy et al., 2009). This limitation is probably due to the difficulty in obtaining a sufficient number of cases for comparison between groups, differences between criteria for defining the cure and sample collection protocols, dependence on the natural occurrence of cases in dairy farms, absence of control groups (without treatment) in commercial farms, and differences in pathogenicity and antimicrobial resistance profile of bacterial isolates, particularly from the Staphylococcus group (Nitz et al., 2020).

Despite the evident effectiveness of extended therapy with conventional protocols, this modality should not be adopted as a rule in dairy cattle breeding, and should be restricted to animals of high economic value, a history of recurrent mastitis caused by refractory agents (mainly staphylococci), due to the higher costs of treatment and increased management of treated animals (Langoni et al., 2017), because the rational use of antimicrobials in animal mastitis is an issue of the One Health approach (Ribeiro et al., 2015).

Staphylococci species are a complex group of bacteria that inhabit the microbiota of the skin and mucous membranes of several domestic animals (Quinn et al., 2011). *S. aureus* and *S. intermedius* are prevalent coagulase-positive staphylococci species involved in bovine mastitis (Wald et al., 2019), particularly *S. aureus* and *S. intermedium* also isolated in the present study. Coagulase-positive staphylococci has been described as the most pathogenic microorganisms related to mastitis in cows and heifers in Canada and the United States (McParland et al., 2019; Campos et al., 2022). This group of bacteria often causes subclinical mastitis, with chronic evolution and refractoriness to conventional antimicrobials. In addition, staphylococci induces an increase in SCC, decreased milk production, and increased rates of early disposal of young animals, comprising a high negative economic impact on the dairy industry worldwide (Wald et al., 2019)

The greater susceptibility of the our staphylococcal isolates to cephalosporins, fluoroquinolones, and aminoglycosides classes of antimicrobials is consistent with similar studies (Oliver et al., 2004; Sharun et al., 2021), which described these drugs as the most effective currently for the treatment bovine mastitis caused by staphylococci during lactation or in the dry period, commercially available for intramammary applications. Cephalosporins are antimicrobials chemically similar to penicillins (beta-lactams), with different generations of drugs and analogs. They have a wide spectrum of action for gram-positive bacteria (including beta-lactamase-producing *S. aureus* strains) and, secondarily, for gram-negative bacteria (Giguère et al., 2013). This group represents the main commercially available antimicrobials for intramammary therapy of mastitis during lactation and/or drying in farm animals, with high effectiveness including β-producing lactamases (Langoni et al., 2017).

In the current study, extended therapy with ceftiofur was carried out on primiparous cows with subclinical mastitis-related staphylococci, where the identification of bacterial growth-inhibiting substances (antimicrobial residues) was investigated using two commercial tests available in Brazil to assess the time needed for the disposal of milk in animals submitted to this prolonged therapy approaches. The presence of antimicrobial residues is a limiting factor for any type of treatment, either intramammary or parenteral, since these components in milk can cause adverse reactions in humans (Sachi et al., 2019). Our findings reinforce this recommendation since the presence of antimicrobial residues was identified in the two commercial tests at the 3 moments of the treated groups. Particularly in M2, more than 90% of the animals were positive for both tests, and even 16 days after the end of therapy (M4), some animals still showed residues in milk.

Thus, use of extended treatment resulted in longer presence of antimicrobial residues in milk samples, a condition that needs to be clarified for producers who choose to use this protocol in the treatment of subclinical mastitis caused by *Staphylococcus* species in dairy cattle.

Sampled animals that developed clinical mastitis were excluded, although SCC greater than 5,000 x10^3 cells/mL was observed in some animals from G2, G3, and G4, confirming a high increase of cellularity, even in subclinical staphylococcal infections. The findings of the present study reinforce that even in animals with a microbiological cure after treatment, the SCC values do not return to levels considered normal (<200x10^3 cells/mL) in a short time because >70% of the microbiological cure rates for the group treated at the beginning of lactation, no animals returned to values below 200,000 cells/mL after three weeks of follow-up. Furthermore, none of the cured animals showed values <200 x10^3 cells/mL after the end of treatment, highlighting the influence on cellularity for a prolonged period in mammary infections by contagious agents, *e.g.,* staphylococci species.

The reduction in CMT scores in cows and primiparous cows treated during lactation have already been previously observed (McDougall et al., 2019). The results obtained in this study confirm the use of SCC as an indicator of subclinical staphylococcal mastitis in dairy herds including primiparous cows, because the milk of a healthy animal, in the first lactation, must have CCS <100 x10^3 cells/mL and, in cows <200,000 cells/mL (National Mastitis Council [NMC], 2006), a fact observed in primiparous cows of G1.

The decrease in milk production in dairy cattle with subclinical mastitis is determined by lesions caused by the pathogens to the epithelial cells of the mammary gland, which reduce the capacity for synthesis and milk secretion. Moreover, the obstruction of ducts in the mammary gland by microabscesses, induced by contagious diseases such as staphylococci may increase the degree of tissue damage and inactivity of the breast parenchyma, reducing milk production (Cheng & Han, 2020).

The lack of speciation by MALDI-TOF MS or other molecular methods for the diagnosis of staphylococci species may be considered limitations of the current study.

Conclusion

Extended intramammary therapy with ceftiofur in bovine primiparous cows subclinically affected by staphylococcal mastitis is effective mainly in cases treated at the beginning of lactation, showing microbiological cure and reduction in SCC, indicating that this alternative therapy approach can be used for the treatment of primiparous cows with staphylococcal infections during lactation.

Animal Ethics and Experimentation Committee

The study was approved by the Animal Ethics and Experimentation Committee (AEEC) guidelines of FMVZ-UNESP/Botucatu, SP, Brazil (number 37-2015).

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