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Factors associated with microbiological and clinical cure of mastitis in dairy cows

[*Fatores associados à cura microbiológica e clínica da mastite em vacas leiteiras*]

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ABSTRACT

709 clinical mastitis cases were analyzed and treated with antimicrobial combination cephalexin-neomycin and the anti-inflammatory prednisolone. A sample of milk was collected to perform a microbiological culture before starting the treatment and 14 days later. Somatic cell count (SCC) was obtained from samples collected on the day of the clinical case (D0), 14 days after (D14) and 28 days after (D28). Of the total, 435 (61.4%) at the D0 exhibited growth of microorganisms. Of the isolated agents, 365 (84%) were Gram-positive, and 66 (16%) were Gram-negative. A clinical cure was achieved in 63% of cases. Bacteriological cure occurred in 75% of cases. Only at D28 after the clinical case a significant SCC reduction was verified. The logistic regression for clinical cure showed significant effects for days in milk and parity ($P< 0.05$). For bacteriological cure, there were significant effects of Log (SCC) D0; clinical cure and quarter affected ($P< 0.05$). In the principal component analysis, the Temperature-Humidity Index was associated with reduced clinical cure of clinical mastitis cases.

Keywords: clinical cure, bacteriological cure, therapy, somatic cell count

RESUMO

Setecentos e nove casos clínicos de mastite foram analisados e tratados com combinação antimicrobiana à base de cefalexina-neomicina e o anti-inflamatório prednisolona. Uma amostra de leite foi coletada para realização de cultura microbiológica antes do início do tratamento e 14 dias depois. A contagem de células somáticas (SCC) foi obtida de amostras coletadas no dia do caso clínico (D0), 14 dias após (D14) e 28 dias após (D28). Do total, 435 (61,4%) no D0 apresentaram crescimento de microrganismos, enquanto em 274 (38,6%) não houve crescimento. Dos agentes isolados, 365 (84%) eram Gram-positivos e 66 (16%) eram Gram-negativos. A cura clínica foi alcançada em 63% dos casos. A cura bacteriológica ocorreu em 75% dos casos. Apenas no D28 verificou-se uma redução significativa na SCC. A regressão logística para a cura clínica mostrou efeitos significativos para dias em lactação e paridade ($P<0,05$). Para a cura bacteriológica, houve efeitos significativos de Log (SCC) D0; cura clínica e quarto afetado ($P<0,05$). Na análise do componente principal, o índice de temperatura-umidade foi associado com a redução da cura clínica dos casos clínicos da mastite.

Palavras-chave: cura clínica, cura bacteriológica, terapia, contagem de células somáticas

INTRODUCTION

Given the complexity of factors related to the occurrence of mastitis in dairy cows and the damages caused by it, the use of antimicrobial therapy is still the main strategy for the treatment of intramammary infections (Roberson, 2012). The limitations to identify the causative agent at the onset of clinical symptoms is one of the reasons for the development of intramammary drugs in combination to cover a wide range of microorganisms, and several therapeutic protocols are frequently performed. This fact

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should be considered since only a small proportion of the herds perform the microbiological culture of clinical mastitis cases and record all cases (Kayitsinga *et al*., 2016).

In order to evaluate the efficacy of intramammary therapy in clinical mastitis cases, the following parameters may be used: clinical cure (CC), somatic cell count (SCC) and bacteriological cure (BC), which the last one is the best criteria for evaluation antimicrobial treatment efficacy (Bradley and Green, 2009). The efficacy in the treatment of clinical mastitis cases is associated to intrinsic factors, pathogen and the therapy used. Factors related to the pathogen comprise resistance to antimicrobial therapy, virulence and pathogenicity (Bradley and Green, 2009). Drug-related factors include route of administration, drug concentration, spectrum of activity and duration of treatment (Hillerton and Semmens, 1999; Bradley and Green, 2009). The factors related to the cow include, for example, SCC, age, stage of lactation, quarter location, parity and effectiveness of the cow's immune response (Bradley and Green, 2009; Pinzón-Sánchez and Ruegg, 2011).

In respect to cows' factors, it is well-known that the immune response has a great impact on the outcome of clinical mastitis. Thus, in the respect of the cow's factors, one well-known factor, especially in tropical climates, that can affect the immune response of dairy cows, and consequently may affect the chance of cure of intramammary infections is the heat stress. Besides this, from another point of view, bacterial pathogens encounter a wide variety of host microenvironments and adjust their virulence to colonize successfully and provoke host pathological conditions. In face of, it is not by chance that climate conditions assume crucial importance in mastitis control programs which indirectly influence the epidemiological triad (host, agent and/or environment). Lastly, identification of the risks factors that affect post-treatment outcomes can help dairy farmers and advisors to select cows that are likely to be cured, or even improve antimicrobial treatment efficacy. For these reasons, identify detrimental factors that can controlled and/or minimized by dairy farmers practices, such as control of heat-stress, may help the improvement of outcomes of clinical mastitis therapy.

Therefore, the factors associated with cure for clinical mastitis of dairy cows treated with intramammary infusion of the antimicrobial combination cephalexin and neomycin and the anti-inflammatory prednisolone were evaluated.

MATERIAL AND METHODS

The Animal Ethics Committee of the Universidade Federal de Minas Gerais (protocol n. 117/2011) approved the protocols for this study.

The study was performed in seven commercial herds in the Midwest region of Minas Gerais - Brazil. The herds were composed of Holstein x Gir cows (½, ¾ and 7/8) submitted on a monthly basis to SCC analysis and individual milk weighing. Herds were between 250 and 700 lactating cows and the production system was semi-intensive. Milking was done twice a day.

Lactating cows that had clinical mastitis (CM) were incorporated into the study only when they were in good general health and had not been treated with antimicrobials in the last 30 days. Clinical cases were classified according to the severity and the intensity of the symptoms according to Pinzón-Sánchez and Ruegg, (2011). Only cows affected with mild and moderate mastitis were analyzed. The occurrence of more than one episode of clinical mastitis in the same quarter in a period equal to or less than 14 days was considered as recurrent mastitis, excluding the animal from the experiment. Information on days in milk, parity and quarter position of the affected animals were collected.

Prior to the experimental period, farm employees were trained to diagnose CM, to classify according to the severity (mild, moderate and severe) and to collect samples for microbiological culture and SCC.

After the CM diagnosis, milk samples were aseptically collected from the affected quarter before starting the treatment and 14 days later to perform the microbiological culture. Milk samples for microbiological examination were frozen at -20°C and analyzed within 30 days after sampling.

For the individual SCC, three samples of milk were collected on the day of the clinical case

(D0), after 14 days (D14) and 28 days after the onset of clinical symptoms (D28), according to the protocol of the National Mastitis Council (Laboratory…, 1999). These samples were taken using a milk collector coupled to the milk-line and stored in 40mL vials containing preservative (2-bromo-2-nitropropane-1,3-diol).
Subsequently, SCC was analyzed using the automated somatic cell count (Bentley 2000, Bentley Instruments Inc., Chaska, USA).

For microbiological analysis, briefly 10μL of each milk sample was plated blood agar plate containing 5% defibrinated sheep blood, followed by incubation at 35°C for 24-48h (Oliver *et al*., 2004). The plates were read, observing the microbial growth, appearance, coloration and number of colonies. In the samples of milk with growth of microorganisms, a representative colony was selected and plated on BHI (Brain Heart Infusion) agar and incubated at 35°C for 24 hours. Subsequently, the isolates were examined by Gram's staining, colony morphology and biochemical tests (Oliver *et al*., 2004). A positive culture was considered when ≥ three colonies were detected, except when the quarters were infected with *Staphylococcus aureus* or *Streptococcus agalactiae*. In that case, they were considered a positive culture when one or more colonies were detected (Blagitz *et al*., 2015). Samples with growth of more than three bacterial species in the microbiological analysis were considered contaminated and excluded.

The clinical cases were treated with intramammary infusion (Masticine-L®, 100mg cephalexin + 100mg neomycin + 10mg prednisolone, Vallée, Montes Claros, Brazil). The treatment initially consisted of applying the medication at each milking until the clinical signs of mastitis disappeared during the three days of treatment (six applications). Animals that show clinical signs during this period were considered clinically uncured, and were excluded from the experiment and treated with the antibiotic routinely used in the farm. In cases which clinical signs disappeared within the first three days of treatment, four more applications (two days) were administered, totaling a maximum of ten applications.

The animals were excluded from the study when the milk sample prior to the treatment was contaminated or had more than one quarter affected. The clinical cases with yeast growth in the pre-treatment sample were excluded from the experiment.

Clinical cure was the absence of clinical symptoms in the affected quarter during the first three days of treatment. The bacteriological cure was evaluated by comparing the results of the pre and post treatment samples. Bacteriological cure was defined when no agent or a different agent was identified in the post-treatment culture. The loss of the post-treatment culture sample resulted in the elimination of the case for bacteriological cure evaluation and only clinical cure was evaluated.

The data of daily humidity, average temperature and precipitation were obtained from the meteorological database provided by the National Institute of Meteorology (INMET) during the experimental period. For the determination of the Temperature and Humidity Index (THI), the following equation was used: $THI = (1.8 \times T + 32) - (0.55 - 0.0055 \times RH) \times (1.8 \times T - 26)$, in which T is the ambient temperature in °C and RH is the relative humidity (A guide …, 1971).

Statistical analyzes were performed using SAS version 9.3 (SAS…, 2011). Initially, descriptive statistics was used to verify data accuracy, to detect missing data and to observe frequency distribution. SCC was log transformed in order to achieve a normal distribution of the data. Chi-square analysis were used to determine the statistical difference between clinical and bacteriological cure rates; quarter affected (front and rear quarters), with significance level of $P \leq 0.05$. The SCC at different times (D0, D14 and D28) was evaluated by Student's t-test with significance level of $P \leq 0.05$. Logistic regression analysis was performed using the experimental data. The variables: bacteriological cure; clinical cure: log (SCC) at D0, D14 and D28; DIM; parity and quarter were analyzed individually to verify the significance and then in combination to evaluate the effect of the individual variables. In the first phase of the analysis, the *P*-values of ≤ 0.20 were selected to the next stage of the analysis. The variables with *P*-values of ≤ 0.05 were maintained in the final model. The principal component analysis (PCA) was performed to evaluate the variables: precipitation; temperature;

relative humidity; THI; Days in milk; Log (SCC) at D0, D14 and D28 in relation to clinical and bacteriological cure. This analysis was based on the correlation matrix.

RESULTS

724 animals had CM during the experimental period. From these, 90% (n= 653) had mild mastitis; 9% (n= 64) moderate and 1% (n= 7) of the animals had severe mastitis. Severe clinical cases were eliminated, remaining 717 quarters. There was a higher frequency of CM in the rear quarters (54%, n= 385) compared to the front quarters (46%, n= 332).

Of the 717 samples analyzed, eight were eliminated from the experimental analysis due to the presence of yeasts (n= 3, 0.4%) and contamination (n= 5; 0.7%), remaining 709 samples. Of the total, 435 (61.4%) presented growth of microorganisms, whereas in 274 (38.6%) no agent was isolated (Table 1). The main isolated agents during the pre-treatment were Gram-positive, representing 84% (n= 365): *S. agalactiae* (n= 112, 30.7%); S. *dysgalactiae* (n= 41, 11.2%); *S. uberis* (n= 27, 7.4%); other *Streptococcus spp*. (n= 107, 29.3%); *Corynebacterium bovis* (n= 32, 8.8%); *S. aureus* (n= 24, 6.6%) and coagulase-negative staphylococci (n= 22, 6.0%).

Gram-negative microorganisms were isolated in 16% (n= 70), mostly *Escherichia coli* (n= 40.57%), *Klebsiella spp* (n= 26.37%) and *Serratia spp* (n= 4.6%). Of the total samples, 435 were used to evaluate the bacteriological cure due to the presence of microorganism isolated in the pre-treatment culture. The bacteriological cure rate was 75.4% (n= 328; Table 1) and higher for Gram-positive microorganisms than Gram-negative ($P \leq 0.001$).

Table 1. Effect of intramammary treatment on the bacteriological and clinical cure of clinical mastitis cases

Pathogens	Bacteriological cure		Clinical cure	
	Total	n (%)	Total	n (%)
No growth	-	-	274	175 (64)
Gram-Positive				
Streptococcus agalactiae	112	102 (91)	112	71 (63)
Streptococcus spp.	107	89 (83)	107	55 (51)
Corynebacterium bovis	32	24 (75)	32	19 (59)
Streptococcus dysgalactiae	41	25 (61)	41	27 (66)
Streptococcus uberis	27	19 (71)	27	26 (96)
Staphylococcus aureus	24	17 (70)	24	23 (96)
Coagulase-negative staphylococci	22	11 (50)	22	10 (45)
Gram-Negative				
Escherichia coli	40	26 (65)	40	25 (63)
Klebsiella spp.	26	11 (42)	26	13 (50)
Serratia spp.	4	4 (100)	4	4 (100)
Total	435	328 (75)	709	448 (63)

There was no difference between SCC at D0 and D14 between the groups (Table 2), but only at D28 a reduction in SCC was observed. There was a similarity in all evaluated groups, except in uncured Gram-negative cases, in which SCC was higher. The SCC of the clinical cases with no bacterial growth (NBG) progressively reduced between D0, D14 and D28. In the Gram-positive group, the SCC reduced only in those cases of bacteriological cure and this reduction was verified at D28. For cured Gram-negative cases, there was SCC reduction only at D28, whereas in uncured mammary quarters there was an increase in SCC verified between D0 and D14, remaining high at D28. The clinical cure rate was 63% (448); no statistical difference was found regarding the clinical cure comparing Gram-positive (63.4%) and Gram-negative (57.6%) microorganisms.

The SCC of clinically cured and uncured cases had a significant reduction at D14 and D28 (Table 3). The SCC of clinically cured animals was different only at D28 in relation to the uncured cases.

Logistic regression for clinical and bacteriological cure is presented in Table 4. Significant effects for clinical cure were DIM (P< 0.001) and parity (P= 0.03). For bacteriological cure, the significant effects were Log (SCC) D0 (P= 0.02), clinical cure (P= 0.001) and quarter affected (P= 0.04).

Table 2. Logarithmic milk somatic cell counts at 0, 14 and 28 days after the clinical case in relation to the results of the pre-treatment samples and bacteriological cure

Result	Status	Day 0	Day 14	Day 28
NBG				
Gram-Positive	Cure	5.74^{aA} ($\pm$0.59)	5.66^{bA} ($\pm$0.71)	5.16^{cB} ($\pm$0.64)
	Uncured	5.62^{aA} ($\pm$0.84)	5.53^{aA} ($\pm$0.62)	5.19^{bB} ($\pm$0.60)
Gram-Negative	Cure	5.43^{aA} ($\pm$0.62)	5.44^{aA} ($\pm$0.67)	5.32^{aB} ($\pm$0.60)
	Uncured	5.67^{aA} ($\pm$0.51)	5.39^{aA} ($\pm$0.81)	5.24^{bB} ($\pm$0.70)
		5.30^{bA} ($\pm$0.64)	5.65^{aA} ($\pm$0.34)	5.65^{aA} ($\pm$0.70)

[a,A]Different letters indicate statistical difference (P≤ 0.05) between row (lower case) and column (upper case), respectively. NBG: no bacterial growth.

Table 3. Logarithmic somatic cell counts at 0, 14 and 28 days after the clinical cure

Result	n	Day 0	Day 14	Day 28
Clinical cure				
Cure	252	5.60^{aA}($\pm$0.74)	5.60^{aA}($\pm$0.61)	5.15^{bB}($\pm$0.61)
Uncured	127	5.54^{Aa}($\pm$0.74)	5.45^{aA}($\pm$0.67)	5.31^{bA}($\pm$0.68)

[a,A]Different letters indicate statistical difference (P≤ 0.05) between row (lower case) and column (upper case), respectively.

Table 4. Logistic regression for variables associated with clinical and bacteriological cure of clinical mastitis under antimicrobial and anti-inflammatory treatment

Variables	Odds ratio	95% CI[3]	*P*-value
Bacteriological cure			
Log (SCC) 0	0.64	0.43 - 0.95	0.03
Log (SCC) 28	1.72	1.06 - 2.78	0.03
Clinical cure	5.75	3.10 - 1.06	0.0001
Quarter[1]	1.92	1.02 - 3.57	0.04
Clinical cure			
Log (SCC) 14	1.49	1.04 - 2.10	0.03
Log (SCC) 28	0.67	0.47 - 0.95	0.03
DIM	0.70	0.58 - 0.84	0.0001
Parity	1.22	1.01 - 1.44	0.03

Log (SCC) 0: log SCC at day 0; Log (SCC) 14: log SCC at time 14; Log (SCC) 28: log SCC at day 28; DIM: Days in milk; CI: confidence interval.

According to Figure 1, in the PCA, the Log SCC D0 variables were directly associated with the clinical cure of clinical mastitis cases and its increase leads to a higher cure rate. Otherwise, the variables THI and DIM were inversely related to the clinical cure of the animals. Therefore, the higher these variables, the fewer clinical cure rates. In relation to the bacteriological cure, the variables average temperature were directly associated, in contrast to humidity and precipitation.

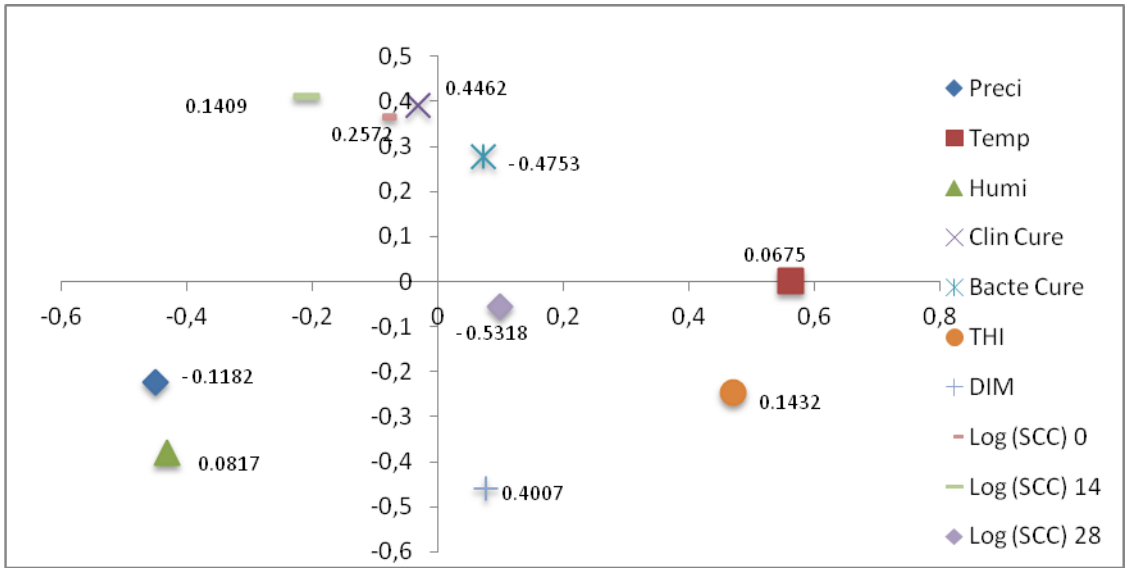

Figure 1. Three-dimensional plot of the principal components analysis (PCA) of the clinical and bacteriological cure of clinical mastitis cases and its correlations with parameters such as precipitation (Preci); Temperature (Temp); Relative humidity (Humid); Clinical cure (Clin cure); Bacteriological cure (Bacte Cure); Temperature and Humidity Index (THI); Days in milk (DIM); Log of the somatic cell count at time 0 (Log (SCC) 0); Log of the somatic cell count at time 14 (Log (SCC) 14) and Log of the somatic cell count at time 28 (Log (SCC) 28). Variables in the same quadrant were closely associated, and those in opposing quadrants had opposite effects (components 1 and 2). The variables in other quadrants were considered as independent variables. Inertia: 57%.

DISCUSSION

Although Gram-negative microorganisms are an important cause of CM (Schukken *et al*., 2011), Gram-positive were the most frequently isolated in the present study (Table 2). Among the isolated microorganisms, there is a higher prevalence of contagious microorganisms, which indicate failures in the control programs for contagious mastitis in dairy farms (Cortinhas *et al*., 2016).

Here, in 39% of CM cases, there was no microbial growth, as previously described (Hoe and Ruegg, 2005; Lago *et al*., 2011). The absence of isolation of mastitis pathogens in milk samples from CM cases may be the result of the spontaneous elimination of pathogens (Smith *et al*., 1985), relatively short duration of infections caused by Gram-negative bacteria; low concentration of microorganisms in milk and pattern of microorganism elimination (Sears *et al*., 1993). In this line of reasoning, the lack of pathogen recovery in the microbiological analysis of milk samples can also be due to the low number of shedding bacteria from clinical mastitis cases associated with the effective control of infection by the cow's immune response (i.e. milk SCC) (Pinzón-Sánchez and Ruegg, 2011) suggesting spontaneous cure of IMI. These hypothesis were reinforced by the progressively reduction in SCC after the clinical case between D0 and D28. An alternative to increase the possibility to identify microorganisms that cause clinical mastitis, which microbiological cultures showed no bacterial growth, is the use of molecular biology. According to Kuehn *et al*. (2013), it is possible to increase the bacteria found in milk from clinical cases with negative isolation by using PCR (real-time polymerase chain reaction), that identifies the microorganisms by the amplification and sequencing of specific regions of the deoxyribonucleic acid (DNA).

The bacteriological cure rate was higher for Gram-positive microorganisms than Gram-negative microorganisms, which are in agreement with previous findings (Lago *et al*., 2011). Although, selective treatment of clinical mastitis was not practiced in the seven dairy farms used here, and in most of the Brazilian

dairy farms. With this in mind, our data strength the possibility to reduce antimicrobial usage in dairy farms by the application of culture-based treatments, which only quarters with CM infected with Gram-positive bacteria will be routinely treated (Lago *et al*., 2011). Otherwise, regarding the high prevalence of CM by contagious pathogens, the expected reduction of antimicrobial usage by the implementation of selective treatment of CM is probably lower in Brazilian dairy herds than other developed countries that have successfully controlled the most important contagious pathogens (i.e. *S. agalactiae* and *S. aureus*).

In mastitis cases in which Gram-positive and Gram-negative microorganisms were isolated, there was a significant SCC reduction at D28 only in cases of bacteriological cure. This is due to the action of the intramammary drug against the microorganism from the mammary gland, resulting in elimination of bacterial infection and reduction of organism response to overcome the infection, with reduction of the inflammatory reaction and SCC. According to Costa *et al*. (2013), the restoration of the mammary gland and the end of the inflammatory reaction lasts from 14 to 21 days after bacteriological cure, which justifies the reduction found in the study only at D28.

In the uncured Gram-positive cases, SCC remained high at all times possibly due to persistence of the pathogen in the mammary gland, favoring the inflammatory process and maintenance of high SCC. Notably, for instance, some udder-adapted *E. coli* strains multiply better in the udder and evade the host cellular innate immune response differently from environmental *E. coli* population, which at least in part may explain our results. Thus, we suppose that udder-adapted *E. coli* population may be less responsive to antimicrobial treatment and persist in the mammary gland, however this hypothesis deserves further investigations. Furthermore, differences in proteins expression among *E.coli* that causes transient and persistent IMIs have been reported (Lippolis *et al*., 2014).

Here, we do not have a control group as this study was carried out in commercial dairy herds – as most mastitis researches are conducted - and because the resistance of dairy farmers to not treat cows with CM. Besides the difficulty in comparing bacteriological and clinical cure among studies due to variations among methodologies (i.e. drug usage, duration of treatment, sampling time), the proportion of bacteriological (75%) and clinical (63%) found here was reasonably consistent with previous findings (Bradley and Green, 2009; Pinzón-Sánchez and Ruegg, 2011; Schukken *et al*., 2013). However, the lack of control group here makes it impossible to establish if successful outcomes were a result of antimicrobial therapy or a result of the cow's immune response against IMI.

High SCC in pre-treatment reduced the chances of CM bacteriological resolution. Melchior *et al*. (2006) reported that chronically infected cows have a worse response to intramammary antimicrobial therapy and, consequently, less chance of bacteriological cure. According to Sol *et al*. (2000) and Bradley and Green (2009) cows with low SCC prior to the onset of clinical symptoms are more likely to experience bacteriological cure than cows with high SCC. The results of the SCC history may be a useful tool before the treatment decision, assessing the possible risks of the procedure.

The affected quarter was considered a risk factor for bacteriological cure. In the present study, there was a higher incidence (P= 0.04) of clinical cases in the rear quarters, in agreement with the study of Dopfer *et al*. (1999). Rear quarters had a lower chance of bacteriological cure possibly due to the higher amount of milk produced, that besides acting as a dilution factor of the constituents in the milk, interfere with the intramammary drug used.

The separate evaluation of temperature and humidity parameters of the environment does not predict thermal stress in cows. THI is used as a tool to better evaluate the influence of environmental variations on cow comfort. Here, to the best of our knowledge, this is the first study that describe the adverse effect of THI for the clinical cure of CM. Heat stress occurs when cows are exposed to ambient conditions with high temperature, high humidity or both, resulting in problems to eliminate excessive metabolic heat. Cows in heat stress have impaired milk production, reproduction and immune function (Liu *et al*., 2014). Impaired immune function may result in reduced

elimination of the CM causative agent, leading to a lower clinical and bacteriological cure rate. In the present study, since the animals live in a tropical climate and are raised on a semi-intensive system, the effects of thermal stress can be even greater due to the incidence of direct solar radiation in the animals. Thus, our data strengthens the idea that climate conditions not only have implications to the incidence of new intramammary infections, but also have a role to the clearance of IMI after antimicrobial treatment. These data support the need to give thermal comfort to dairy cows with clinical mastitis in attempt to reduce the detrimental effect of adverse climate conditions (i.e high THI), and consequently improve the efficacy of antimicrobial therapy.

CONCLUSIONS

The Gram-positive microorganisms occur with high frequency in CC of mastitis and the intensification of mastitis control programs in farms is fundamental. For the cured animals, there was a reduction in SCC 28 days after the clinical case. The history of SCC, DIM and PA are useful and user-friendly tools for decision-making before conducting mastitis CC treatment, assessing the chances of success of the procedure. It is of interest to provide thermal comfort for dairy cows with clinical mastitis in an attempt to reduce the damaging effect of thermal stress on the animal's immune response (i.e., elevated THI) and hence improve the effectiveness of the antimicrobial therapy used.

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