MICROBIOLOGICAL PROFILE OF MILK PRODUCED ON DAIRY FARMS IN THE STATE OF SÃO PAULO ACCORDING TO THE STANDARDS OF NORMATIVE INSTRUCTION 62 OF THE BRAZILIAN MINISTRY OF AGRICULTURE, LIVESTOCK AND FOOD SUPPLY

[*Perfil microbiológico do leite de propriedades paulistas em relação às condições exigidas pela Instrução Normativa 62 do MAPA*]

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RESUMO – Objetivou-se avaliar o atendimento à Instrução Normativa 62 em propriedades leiteiras no estado de São Paulo, estudar a ocorrência de mastite subclínica de quartos mamários por meio do *California Mastitis Test* (CMT) e da contagem de células somáticas (CCS), estabelecer a etiologia da mastite, analisar o comprometimento da CCS das diferentes propriedades de acordo com os micro-organismos isolados e verificar a influência das condições climáticas na ocorrência de mastite. Os percentuais de mastite subclínica analisados pelo CMT em relação ao número de animais e quartos mamários foram de 48,3% (232/480) e 26,2% (496/1892) respectivamente. Considerando o limite atual de CCS da IN 62, 88,8% das propriedades atendem a IN 62. No entanto, analisando o objetivo final da IN 62 que é atingir 400.000 céls/mL algumas propriedades já ultrapassam e outras estão muito próximas desse limite. As frequências dos micro-organismos por propriedade foram diferentes. As propriedades E e D tiveram os resultados mais críticos. Entre as 388 amostras positivas ao exame microbiológico, *Staphylococcus aureus* foi o micro-organismo mais prevalente, com 34,02% dos isolados. O *Streptococcus* spp. foi o agente mais associado às elevadas CCS, com coeficiente de correlação de 0,20286. O trabalho mostrou que os patógenos da mastite consistem em um fator de variação da resposta celular. Os micro-organismos isolados representam uma redução na produção e qualidade do leite, além de oferecerem vários riscos à saúde humana. A associação entre clima e mastite está subordinada ao tipo de manejo adotado, profilaxia e também à estrutura e limpeza das instalações da propriedade.

Palavras-Chave: *Staphylococcus aureus*; contagem de células somáticas; mastite bovina; Instrução Normativa 62.

ABSTRACT – The objectives of this study were to determine whether dairy farms in the State of São Paulo meet the Normative Instruction 62 (NI-62), to evaluate the occurrence of subclinical mastitis in mammary quarters by the California Mastitis Test (CMT) and somatic cell count (SCC), to establish the etiology of mastitis, to analyze SCC problems on the different farms according to the microorganisms isolated, and to determine the influence of climatic conditions on the occurrence of mastitis. The incidence of subclinical mastitis determined by the CMT according to the number of animals and mammary quarters analyzed was 48.3% (232/480) and 26.2% (496/1892), respectively. Considering the SCC limit current NI-62, 88,8% of the farms meet the standards of NI-62. However, considering the final objective of NI-62 which is to achieve 400.000 cells/mL, some farms already exceed this limit. The frequency of microorganisms differed between properties, it was observed the most critical effects on farm E and D. *Staphylococcus aureus* was the most common microorganism isolated from the 388 samples positive in microbiological test, accounting for 34.02%. *Streptococcus* spp. was the agent most associated with elevated SCCs, with a coefficient of correlation of 0.20286. This study showed that mastitis pathogens are responsible for variations in the cellular response. The microorganisms isolated cause a reduction in production and milk quality, in addition to posing a risk to human health.

Keywords: *Staphylococcus aureus*; somatic cell count; bovine mastitis; Normative Instruction 62.

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INTRODUCTION

Mastitis is a disease that affects the udder of cows and substantially impairs the maintenance of milk quality. The etiology of mastitis is diverse and complex, but the most important cases are those caused by microorganisms (Krewer et al., 2013). The most common contagious agents isolated from mastitis cases are *Staphylococcus aureus* and *Streptococcus agalactiae*. The main reservoir of these pathogens is the udder of an infected animal and infections are spread from cow to cow or to uninfected mammary quarters during milking by contaminated equipment, milkers' hands, or towels used in more than one cow (Fonseca & Santos, 2007).

In Brazil, quality milk production is laid down in Normative Instruction No. 62 (NI-62) from December 29, 2011, which establishes minimal standards for the commercialization of milk (Oliveira et al., 2011). In an attempt to control mastitis on dairy farms and to produce high-quality milk, it is necessary to establish an action plan based on the diagnosis of the situation on each property (Odair, 2013). The elaboration of on action plan requires knowledge of the epidemiology of the main mastites-causing microorganisms, the measurement of somatic cell count (SCC), and the inclusion of the California Mastitis Test (CMT) in daily routine. The CMT is a useful screening test for the detection of subclinical mastits that can be easily applied and interpreted. Somatic cells are desquamated cells of the glandular epithelium and leukocyte lineage which are responsible for the defense of the organism against infection. The results obtained with the investigation of mastitis-causing agents are fundamental to understand specific problems of herds, to guide rational mastitis control measures, and to suggest changes in the adopted management (Saab et al., 2014).

Therefore, the objectives of this study were to evaluate the compliance of nine dairy farms in the State of São Paulo with the requirements of NI-62, to investigate the occurrence of subclinical mastitis in mammary quarters by the CMT and SCC, to establish the microbiological profile of mastitis-causing agents in milk samples obtained from the mammary quarters of lactating cows, to analyze SCC problems on the different farms according to the microorganisms isolated.

MATERIAL AND METHODS

Milk from nine dairy farms (designated by letters A to I) producing refrigerated raw milk and type A (produced, processed and bottled on the farm, milking should be compulsorily mechanic with channeling milk in a closed circuit and cooling in tanks to a maximum of 4ºC and obeying the 400.000 SCC limit), located in the State of São Paulo, was evaluated. On each farm, once every biweekly milk samples (one daily sampling) were collected over a period of 2 months from 15 randomly selected cows, which were representative of the herd, a total of 540 animals and 2160 mammary quarters. Table 1 shows the characteristics of each of the farms.

Sample collection was performed following strict procedures of antisepsis of the mammary quarters. The samples were stored in sterile test tubes, previously identified with the number of the animal and mammary quarter.

All mammary quarters of cows participating in the sampling were submitted to the CMT, except for farm B which has its own system in which somatic cells are determined simultaneously during milking. Because of that were submitted to the CMT 480 animals and 1892 mammary quarters. Milk samples for SCC analysis were collected into sterile flasks from the balloon collectors or from the cans themselves (in the case of mechanical/bucket milking). Somatic cell counting was performed by flow cytometry in a 52 Somacount 300®. The microorganisms were isolated and identified at the Laboratory of Milk Quality, Centro APTA Bovinos de Leite, Instituto de Zootecnia.

Table 1. Main characteristics of production of dairy farms, designated from A to I.

Characteristics	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
Milk	Refrigerated raw	Type A	Refrigerated raw	Refrigerated raw	Refrigerated raw	Refrigerated raw	Refrigerated raw	Refrigerated raw	Refrigerated raw
Breed	Holstein	Holstein	Holstein/Jersey	Mixed/Jersey	Mixed/Holstein	Girolando	Mixed/ Jersey	Mixed	Mixed/ Holstein
Number of lactating cows	56	1450	59	27	110	55	29	17	249
Daily milk production (L)	650	42000	1660	370	1500	800	667	220	4000
Type Milking	Mechanical/ *Tandem*	Mechanical	Mechanical/ *Tandem*	Mech./Bucket to Foot	Mech./ Fishbone	Mech./Bucket to Foot	Mechanical/	Mech./Bucket to Foot	Mech./ Fishbone
Number of milkings	2	3	3	2	2	2	2	2	2
Veterinary assistance	Constant	Reproductive area	Constant	None	None	None	Constant	None	Reproductive area
Hygiene of milking facilities	Satisfactory[1]	Satisfactory [1]	Satisfactory[1]	Unsatisfying[2]	Unsatisfying[2]	Satisfactory [1]	Satisfactory[1]	Unsatisfying	Satisfactory [1]
Hygiene of milking equipment	Automatic cleaning	Automatic cleaning	Automatic cleaning	Manual and poor cleaning[3]	Automatic cleaning	Automatic cleaning	Automatic cleaning	Manual cleaning, but adequate[4]	Automatic cleaning
Cooling Milk	Cooling tank	Cooling tank	Cooling tank	Cooling tank	Cooling tank	Cooling tank	Cooling tank	Cooling tank	Cooling tank
Tamis test	Yes	Yes	Yes	No	No	Yes	Yes	No	Yes
Pre-dipping	No	Yes	Yes	No	No	Yes	Yes	No	Yes
Teats drying	Paper disposable towel	Paper disposable towel	Paper disposable towel	No	No	Paper disposable towel	Paper disposable towel	Paper disposable towel	Paper disposable towel
Post-dipping	Yes	Yes	Yes	No	Yes	Yes	Yes	No	Yes
Waiting room	Yes	Yes	Yes	No	Yes	No	Yes	No	Yes

Hygiene satisfactory milking facilities [1] – Good hygiene, proper use of products and procedures; Unsatisfactory hygiene of milking facilities[2] - Failures in hygiene, incorrect use of products and procedures; Manual and poor cleaning[3] - Weaknesses in clean condition of the milking equipment, lack of cleaning and sanitizing; Manual cleaning, but adequate [4] - Cleaning correct resulting from a physical action performed well and immediately after milking (thus totally removing milk residues, mainly fat, bacteria or yeasts) and obeying a suitable sequence.

RESULTS

The occurrence of subclinical mastitis determined by the CMT is shown in Table 2.

Table 3 shows the mean, median, range and standard deviation of SCCs obtained for each of the nine dairy farms participating in the study, as well as the results of analysis of variance.

Table 2. Distribution of subclinical mastitis in lactating cows and mammary quarters of animals from nine dairy farms in the State of São Paulo

	Cows				Mammary quarters			
	Negative CMT		Positive CMT *		Negative CMT		Positive CMT	
Farm	n	%	n	%	n	%	n	%
A	40	66.7	20	33.3	204	87.2	30	12.8
B	NA	NA	NA	NA	NA	NA	NA	NA
C	40	66.7	20	33.3	211	88.3	28	11.7
D	23	38.3	37	61.7	153	64.0	86	36.0
E	12	20.0	48	80.0	100	41.7	140	58.3
F	27	45.0	33	55.0	160	66.7	80	33.3
G	33	55.0	27	45.0	171	72.5	65	27.5
H	32	53.3	28	46.7	184	81.4	42	18.6
I	41	68.3	19	31.7	213	89.5	25	10.5
Total	248	51.7	232	48.3	1396	73.8	496	26.2

CMT: California Mastitis test; NA: not available.*At least one positive mammary quarter in the CMT.

Table 3. Mean somatic cell count (x 10^3 cells/mL) of milk samples obtained from the balloon collectors or cans of lactating cows on nine dairy farms in the State of São Paulo

Farm	Geom. mean (x10^3 cells/mL)	Transf. mean (log10)	Median (x10^3cells/mL)	Maximum (x10^3cells/mL)	Minimum (x10^3cells/mL)	SD
A	29.2^{b}	1.50^{e}	24.5	9999	1	1302
B	114.4^{b}	2.10^{d}	141.5	6290	11	966
C	203.0^{b}	2.30^{cd}	183.0	2027	25	388
D	495.9^{b}	2.70^{b}	475.5	7069	28	1543
E	$1.213.0^{a}$	3.10^{a}	1537.0	7440	69	1812
F	304.0^{b}	2.50^{bc}	346.5	7623	8	1844
G	398.5^{b}	2.60^{bc}	363.0	5716	37	934
H	215.7^{b}	2.33^{cd}	279.5	2839	8	760
I	100.6^{b}	2.00^{d}	80.5	1723	14	346

Means in the same column followed by the same superscript letter do not differ significantly by the Tukey test (P = 0.05). Geom. Mean: general geometric mean of each farm; Transf. mean: transformed mean; SD: standard deviation.

Pathogen growth was observed in 388 (18.4%) of the 2,115 milk samples submitted to microbiological testing. Analysis of variance and the Tukey test showed an effect of farm ($P < 0.05$), i.e., the farms influenced the results of the absence of microorganisms. The largest number of positive samples was observed on farms D, E and G (42.9, 28.0 and 27.5%, respectively). On farm F, the percentage of positive results in the microbiological test was 9.3%. Farm I showed the lowest bacterial growth, with 96.6% of the samples being negative, followed by farms H (91.5%), B (90.0%), C (86.1%), and A (79.1%). Figure 1 illustrates the percentage of each microorganism isolated on the farms in relation to the total number of positive samples in the microbiological test.

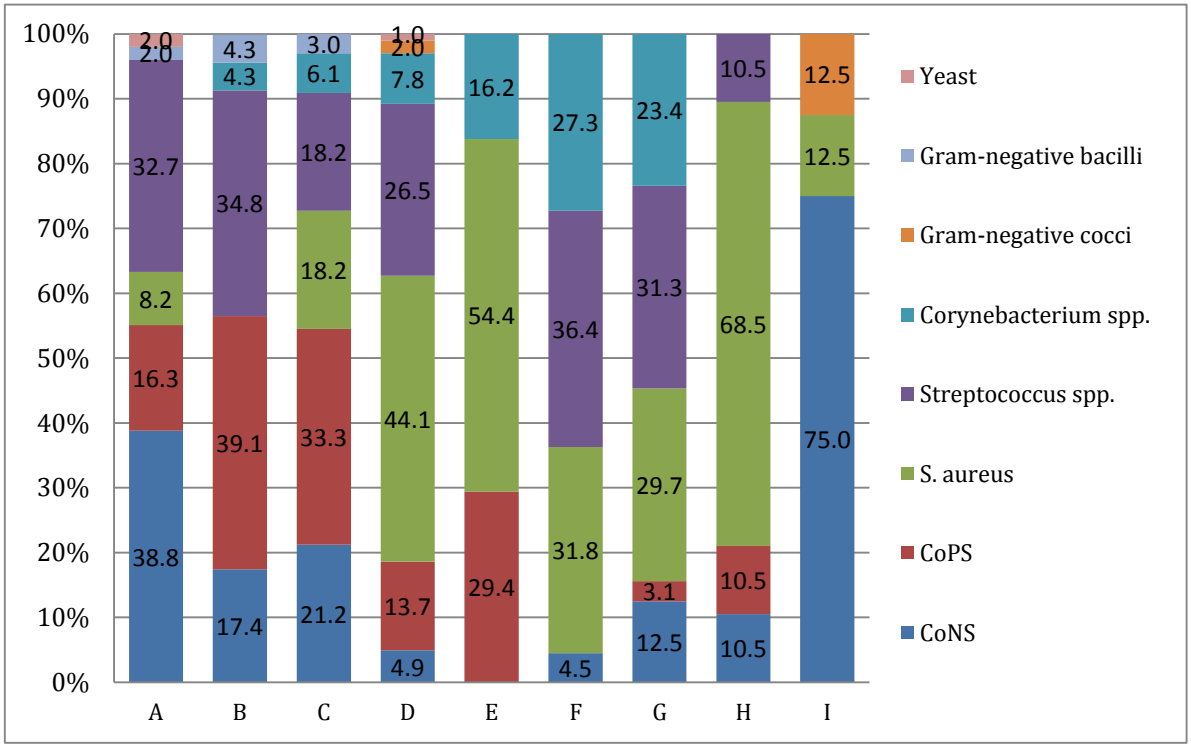

Figure 1. Relative frequency of isolation of each microorganism from mammary quarter milk samples in relation to the total number of positive samples on each of the nine farms studied in the State of São Paulo, Brazil. CoNS: coagulase-negative staphylococci; CoPS: coagulase-positive staphylococci; *S. aureus*: *Staphylococcus aureus*; *Strepto*.: *Streptococcus* spp.; *Coryne*.: *Corynebacterium* spp.

The frequencies of microorganisms was significantly different between farms ($p < 0.05$), except for farms B and C which showed no significant difference in the frequency of microorganisms.

Staphylococcus aureus accounted for 34.0% of all isolates. *Streptococcus* spp. were the second most common agents (22.4%), followed by CoPS and CoNS. *Corynebacterium* spp. were the least frequent. Traces of microorganisms included Gram-negative cocci (accounting for 0.2% of all samples analyzed and for 0.8% of all positive samples), Gram-negative bacilli (the same frequencies as cocci), and yeast (0.1% of all samples analyzed and 0.5% of all positive samples), which were not expressively relevant.

Pearson's correlation test and hypothesis testing of null correlation provided moderate coefficients. Among the microorganisms analyzed, *Streptococcus* spp. showed the most significant correlation with high SCC (0.20286).

DISCUSSION

The CMT results of the mammary quarters (Table 2) showed wide variation in the occurrence of subclinical mastitis, ranging from 10.5 to 58.3%. This discrepancy has also been reported in the study of Oliveira et al. (2010) in which a positive test result ranged from 12.5 to 73.3% in 10 different dairy herds. The highest percentage of positive results was observed on farm E, i.e., 58.3% of all mammary quarters had a score of 1, 2 or 3 in the CMT. Considering the CMT of lactating cows, 80% of the animals of this farm had subclinical mastitis, which is a matter of concern. This result is consistent with the inadequate management practices, the presence of pets moving around freely in the milking room, and deficient hygiene standards observed on this farm (washing the teats with water and soap without drying instead of correct pre-milking teat dipping). In the present study, the relative frequencies of potential cases of subclinical mastitis according to the number of animals and mammary quarters were 48.3% (232/480) and 26.2% (496/1892), respectively. These frequencies are higher than those obtained by Oliveira et al. (2011) in the region of Rondon do Pará, State of Pará (15.6% and 6.6%). In the study of Langoni et al. (2011) evaluating 10 dairy farms in different regions of the State of São Paulo, 19.27% of the samples tested were positive for subclinical mastitis.

The percentage of positive animals (232/480, 48.3%) was similar to that reported by Oliveira et al. (2010) in a study evaluating 187 cows from 10 dairy farms in the Ilhéus-Itabuna micro-region, State of Bahia, in which 72 (38.5%) samples were positive for subclinical mastitis. The highest

frequencies of positive animals were 61.7% and 80% on farms D and E, respectively. The highest percentages of negative mammary quarters in the CMT were observed on farms A, C and I (87.2, 88.3 and 89.5%, respectively). These rates indicate good control of mammary gland health in the respective herds. According to Fonseca & Santos (2007), the average incidence of subclinical mastitis on milk-producing farms in Brazil is about 40% and the objective of adequate mastitis control is to achieve levels of less than 15%.

With respect to SCC data (Table 3), an effect of property ($p < 0.05$) was observed in both analyses of variance (using geometric means and log10 transformed means), i.e., the farm influenced SCCs. However, the analysis using log10 data was more consistent in indicating statistically significant differences. The farm that most exceeded the SCC limit established by NI-62 was farm E, with a general geometric mean of 1,213,000 cells/mL ($p < 0.05$). It should be noted that this farm exhibited the most critical production characteristics. The second highest mean SCC was obtained for farm D. This SCC result agrees with the frequencies of a positive CMT.

Among the herds studied, the lowest SCC was obtained for farm A, followed by farm I, farm B and farm C.

It is important to note that the last two farms (B and C) were equipped with the most adequate structures for any adverse climatic conditions, providing better natural conditions of comfort, ease of management, animal movement, and machines and equipment in a rational and economic manner, thus facilitating the whole process of milk production and preservation. Farm D exhibited a mean SCC that exceeds the final limit of NI-62 which will come into effect on June 1, 2016 in the Southeastern, South and Midwest region (400,000 somatic cells) for refrigerated raw milk. Farms F and G were close to this limit. The geometric mean of farm H, was also slightly altered, calling attention to the fact that a mean SCC higher than 200,000 cells/mL is a strong indicator of subclinical mastitis on the property (Smith, 2002; Brito et al., 2012[a]). Thus, it can be inferred that only farms A, B, C and I, which accounted for 44.4% of the farms studied, were effectively close to this threshold, i.e., a reliable level of health.

The herds of the farms with considerable SCCs had precarious management conditions and no veterinary care. Farms D, E and H do not perform the Tamis test, maintain unsatisfactory hygiene standards of the milking facilities, do not perform pre-milking teat dipping, and do not cull animals with chronic mastitis, factors that favor the infection of healthy cows (Brito et al., 2005). On farms D and E, the performance of the workforce is unsatisfactory and the teats are not dried after washing with water and soap. Farm E neglects milking management, with pets moving around freely in the milking room, among other disconformities. Farms F and H do not possess a waiting room, a fact that contributes to the release of oxytocin which, in turn, stimulates milk let-down, preventing the retention of residual milk (Costa, 2008). According to Rosa et al. (2009), the abnormal accumulation of milk in the udder favors the propagation of microorganisms, predisposing to the occurrence of mastitis. Farms D and H do not perform post-milking teat dipping. Additionally, the milking equipment of farm D is cleaned manually and in a precarious manner. All of the factors cited play an important role in increasing the SCC in lactating cows. Miller and Nesi (2012), collecting samples on 23 dairy farms in the municipality of Ipuaçu, Santa Catarina, observed 73.91% of the samples to be above the limit established by NI-51 (in effect during that period). Therefore, as found in the present study, the disapproval rates of the farms was related to management failures at the time of milking and improper cleaning of the milking environment and equipment, in addition to the lack of knowledge of producers regarding prophylactic measures that reduce contamination of the mammary gland with pathogens. Analysis of the logarithmic data by the Tukey test showed no significant difference between farms B, C, H and I ($p < 0.05$).

Farm A differed significantly from all other farms ($p < 0.05$), with the results indicating the best control of SCCs on this farm. However, this low SCC may have been influenced by the most prevalent microorganisms, which were subsequently identified as CoNS, since this group is frequently associated with a moderate increase in SCC (De Vliegher et al., 2003). The microorganisms that can be isolated from the skin of the udder, the teat canal and the environment, differ in epidemiology, virulence and response to treatment. The description of pathogenicity can vary from protectors other mastitis-causing bacteria, being indifferent in udder health, or be causing mastitis (De Vliegher et al., 2012). Classically, the causative agents of mastitis can be classified as primary and secondary pathogens. The most common major pathogens include *S. aureus*, *S.agalactiae*, *coliforms*, streptococci and enterococci environmental origin. Among the secondary pathogens include *Staphylococcus* sp. coagulase negative and *Corynebacterium* sp. This classification takes into account the fact that the main pathogens are considered as such, since they result in major changes in milk composition and SCC (Souza et al., 2009).

Farm B (114,400 cells/mL), the only dairy farm of this study producing whole milk type A, is in accordance with NI-62 which establishes a limit of 400,000 cells/mL for this category until June 30, 2016, for the Southeastern, Southern and Midwest regions. The farms with the lowest SCCs, i.e., farms A, I, B and C, had adopted administrative management tools. Additionally, both employees and owners had satisfactory technical knowledge of the milking routine and mastitis when compared to the farms with higher SCCs.

Eight (88.8%) of the nine farms studied met the current limit established by NI-62 (500,000 cells/mL), which will be in effect until June 30, 2016. It should be noted that farm D, with a mean of 495,900 cells/mL, is already close to this limit. Similar results have been reported by Langoni et al. (2011) who evaluated 10 farms in different regions of the State of São Paulo. On the basis of the average SCC of the animals, eight farms (80%) were in accordance with the limit determined by NI-51 which was < 750,000 cells/mL at that time. According to the authors, if the limit set to be adopted later were considered (< 400,000 cells/mL), only one of the 10 farms would be adequate. The authors also emphasized that the prevalence of subclinical mastitis was high on four farms (40%) when considering a normal limit of up to 15% in the herd. In the study of Ribeiro et al. (2012) analyzing samples from 25 dairy farms in the metropolitan region of Porto Alegre, Rio Grande do Sul, 53.2% of the farms were within the limit established by NI-62.

The results of the bacteriological tests revealed a significant number of infected animals. On farm A (Figure 1), 20.9% of the samples submitted to microbiological analysis were positive, i.e., contained the etiological agents of mastitis. Coagulase-negative staphylococci were the most frequent microorganisms (38.8% of all isolates), followed by *Streptococcus* spp. (32.7%). This farm had the lowest mean SCCs.

On farm B, only 10% of the samples analyzed tested positive. The most frequently isolated agents were CoPS, which accounted for 39.1% of all positive samples, and *Streptococcus* spp. (34.8%). The mean SCCs of this farm were among the three lowest counts.

Coagulase-positive staphylococci were the most frequently isolated agents on farm C, accounting for 4.7% of all samples analyzed and for 33.3% of all positive samples. The second most common agents were CoNS (21.2%), followed by *S. aureus* and *Streptococcus* spp. with the same frequency of isolation. The SCC on this farm was generally under control, with an overall mean of 203,000 cells/mL. Farms B and C, together with farms A and I (described below), had appropriate milking management practices, including procedures of teat disinfection before milking, stimulation of milk ejection, efficient and rapid milk extraction, and teat disinfection after milking. According to Fonseca & Santos (2007), the combination of these procedures constitutes the most effective strategy to prevent the transmission of contagious agents and, to a lesser extent, of environmental agents during milking. Furthermore, the decisive role of the milking unit manager in mastitis control programs should be emphasized. This professional is a key element in the adequate implementation of milking procedures, with some of them appearing to ignore some points of correct management on the remaining farms.

The highest percent growth of pathogens was observed on farm D where 42.9% of all samples analyzed were positive. This finding is extremely important and serves as a warning regarding the management adopted and the precarious hygiene standards throughout the milking procedure. The agent showing the highest prevalence was *S. aureus* (44.1% of all positive samples), followed by *Streptococcus* spp. (26.5%). The SCC observed on this farm was the second highest count, with an overall mean of 495,900 cells/mL. A count of 950,500 cells/mL was obtained during the third sampling. This result should increase the attention of dairy farm owners to determine, together with their milking unit managers, what is causing this alarming increase in SCC. The isolation of *S. aureus* and *Streptococcus* spp. as the most common agents indicates that both species were related to the cause of the high SCC, particularly *Streptococcus agalactiae* which has been associated with peaks in SCCs in different studies (Oliver et al., 2004).

The most worrisome results were obtained for farm E. *Staphylococcus aureus* was the most frequently isolated agent, accounting for 15.2% of all samples analyzed and for 54.4% of all positive samples, followed by other CoPS (29.4%). According to a study by Middleton (2013) conducted in the United States, *S. aureus* mainly causes subclinical intramammary infections that often become chronic. Furthermore, these infections are frequently refractory to antimicrobial treatment. The economic impact of mastitis caused by *S. aureus* generally manifests through an increase in the SCC of milk and a reduction in production. However, regional differences and differences between herds in terms of the impact of intramammary infections caused by *S. aureus* exist and this agent can be associated with a high incidence of clinical disease in some cases. The SCC of this property was expressively above the

limit determined by the current NI-62 in all four samplings. The overall mean on this farm was 1,213,000 cells/mL. Interestingly, this was the only farm on which no CoNS were isolated. These findings agree with the literature indicating CoNS to be associated with moderate increases in SCC.

The most prevalent genus on farm F was *Streptococcus* spp. (36.4%), followed by *S. aureus* (31.8% of all isolates) and *Corynebacterium* spp. (27.3%). The SCC of this farm was the fourth highest count among the nine farms studied, demonstrating that *Streptococcus* spp. was intimately related to the increase in SCC. It should be noted that a geometric mean of 817,200 cells/mL was obtained during the first sampling on this farm.

The profile of isolation on farm G was similar to that observed on farm F. *Streptococcus* spp. were the agents most frequently isolated on this farm, accounting for 31.3% of all positive samples in this herd. The second most common agent was *S. aureus* (29.7) and, as observed on farm F, the third most common agents were *Corynebacterium* spp. (23.4% of all isolates). The SCC of this farm was the third highest count, a finding supporting the association between *Streptococcus* spp. and high SCCs. According to Souza et al. (2009), the genus *Streptococcus* spp., more specifically the contagious species *S. agalactiae*, is associated with increases in SCC.

Farm H was the property with the second smallest number of positive samples in the microbiological test. The most frequently isolated agent was *S. aureus* which accounted for 68.% of all positive samples. The same frequency of isolation was observed for CoNS, CoPS and *Streptococcus* spp. (10.5% each of all isolates). The overall mean SCC of this farm was 215,700 cells/mL. This may be due to this property of having the smallest number among all animals, and thus have reduced transmission cow to cow.

On farm I, CoNS were the most common agents, but their frequency of isolation was low. This farm had the lowest percentage of positive bacteriological tests (3.4%) among all farms studied, an important and encouraging finding. The SCC of this farm was one of the lowest, second only to farm A. It was noted in the present study that farms on which the CoNS group was the most common microorganism had the lowest SCCs. This result agrees with Schukken et al. (2009) who stated that the increase in SCC is usually moderate in cows with mastitis caused by CoNS. However, these authors also emphasized that the high prevalence of these pathogens in dairy herds may result in considerable increases in the SCC of tank milk. Coagulase-negative staphylococci have been indicated as the main causative agent of mastitis in first-lactation cows, suggesting that these agents could be responsible for possible reductions in milk production (Dufour et al., 2012).
Staphylococcus aureus was the most frequently isolated agent (34.0% of all positive samples) and wasthe most common microorganism on three farms. This finding is a matter of concern since *S. aureus* is associated with cases of greater pathogenicity (Hertl et al., 2014). *Staphylococcus* spp. thus continue to be the agents most frequently isolated from this type of infection and play an important epidemiological and clinical role in bovine mastitis.

The farm with the highest SCC had *S. aureus* as the most common contagious agent. This result contrasts in part with reports in the literature which show *Streptococcus agalactiae* to be the microorganism associated with high SCCs (Coser et al., 2012; Coutinho, 2014). However, *Streptococcus* spp. coexisted with *S. aureus* on most of these farms with high SCC, a fact that does not rule out the association between *Streptococcus* spp. and the high SCCs observed.

With respect to CoNS, the result of the present study agrees with the findings of Mahzounieh et al. (2003) who reported a percentage of 9.04% (36 samples) of CoNS among 398 samples collected from dairy herds in Iran, Arak region, which were positive in the microbiological test. Mota et al. (2012) analyzed 740 samples collected on farms in municipalities of the State of Pernambuco (Recife region, Agreste and Zona da Mata) that tested positive by microbiological analysis; of these, 291 (39.3%) were bacteria of the genus *Staphylococcus* spp. and CoNS were the most common, detected in 170 (58.4%) samples.

The highest percentage of CoPS was observed on farm E, followed by farm D. The presence of this group of microorganisms may have been favored by the management and hygiene failures observed on these farms. No statistically significant difference was observed between farms A, B and C. Santos (2008) found higher percentages than those observed in the present study (17% of all isolates) in a study including 35 dairy herds located in the southern region of the State of Minas Gerais, in which 1,700 samples were positive in the microbiological test. Coagulase-positive staphylococci were the main pathogens present, which accounted for 34.29% of all isolates.

It should be noted that in the present study 250 (64.4%) of the 388 samples that were positive in the microbiological test were bacteria related to the genus *Staphylococcus*; of these, 66 (26.4%) were CoPS, 52 (20.8%) were CoNS, and 132 (52.8%)

were *Staphylococcus aureus*. These data confirm that bacteria of the genus *Staphylococcus* are the main etiological agents of infectious bovine mastitis (Lange et al., 2011). In a similar study conducted by Mota et al. (2012) on 15 dairy farms in the State of Pernambuco, the percentage of bacteria of the genus *Staphylococcus* was 39.3%, and 12.7% of these bacteria were CoPS.

The Tukey test revealed no difference in the frequency of *S. aureus* between farms D and E. These results are consistent with the characteristics of these farms where management deficiencies and failures were observed. Infections arising from sources such as the milking machine, air, the animal's skin and humans are factors that contribute to the maintenance of mastitis caused by *S. aureus* in the herd (Zadoks et al. 2002).

The present results indicate *S. aureus* as the most common microorganism. This result is in accordance with those reported by Chagas et al. (2012) who found *S. aureus* to be the most prevalent agent among 137 milk samples showing microbial multiplication collected on a farm in Minas Gerais, which accounted for 45.2% (62 samples) of all isolates. The authors observed inappropriate cleaning of the milking equipment by the employees on the days of sample collection, and treatment of dry cows was not effectively performed by the employees. The present results confirm the findings of Voltolini et al. (2001) who reported *S. aureus* to be the main agent responsible for cases of contagious mastitis in dairy herds, which were characterized by a large number of cases of subclinical infection and sporadic episodes of clinical mastitis.

There was a significant difference at the 5% level in the presence of *S. aureus* between samplings on the different farms. Similar results have been reported by Zafalon et al. (2008) who performed 16 samplings in a dairy herd located in the State of São Paulo and observed a probable influence of the environment on the higher prevalence of *S. aureus*. The authors suggested the possible occurrence of a mutation or greater dispersal of strains in the environment originating from other sources of contamination.

With respect to *Streptococcus* spp., farm D exhibited the highest frequency of these microorganisms, followed by farms G and A. As can be seen in Figure 1., these microorganisms were the most prevalent on farms G and F, indicating that the significantly elevated SCCs on these farms were caused by *Streptococcus* spp. (farm F exhibiting an overall geometric mean of 348,100 cells/mL and exceeding the mean of 800,000 cells/mL in the 1st sampling). *Streptococcus* spp. accounted for 22.4% of all isolates and were the second most prevalent agents. This result agrees with the findings of Lakew et al. (2009) who studied dairy herds from Asella, southeastern Etiopia. In that study, 24.8% of the 133 samples that were positive in the microbiological test were *Streptococcus* spp., preceded only by *Staphylococcus* spp.(41.4%). Like in the present study, the authors attributed the high prevalence of contagious mastitis to poor hygiene conditions and inadequate management of clinically infected cows. The high prevalence of bacteria of the genus *Streptococcus* spp. observed in this study is not a recent finding. According to Santos et al. (2007), this genus is commonly isolated from mammary gland infections in cattle. These authors also emphasized that the control of *Streptococcus* spp. has been difficult because of the wide distribution and inaccurate description of species, a fact impairing epidemiological studies.

Farm G exhibited the highest frequency of *Corynebacterium* spp., followed by farms D, E and F which were not significantly different from each other. In the present study, *Corynebacterium* spp. accounted for 11.08% of all positive samples in the microbiological test and was the fifth most common microorganism in terms of the number of isolates. In the study of Krewer et al. (2013) evaluating eight dairy farms, *Corynebacterium* spp. accounted for 35.3% of a total of 527 isolates, the second largest number of isolates after *Staphylococcus* spp. (49.1%). *Corynebacterium* spp. are generally considered secondary pathogens that mainly infect cows during lactation without causing clinical signs and are associated with moderate increases in SCC. Mastitis caused by these microorganisms is usually characterized by mild clinical signs and subtle alterations in the physicochemical properties of milk (Martins et al., 2010).

Mixed infection was observed in three (0.77%) of the samples that exhibited bacterial growth. A higher mixed infection rate of 15.5% was found by Souza et al. (2009) among isolates obtained from herds in Rio de Janeiro and Minas Gerais. According to Langoni (2013), etiological plurality is well known in mastitis.It can thus be noted that the use of correct techniques of milking hygiene and equipment improves compliance of producers with normative instructions, which does not occur with the simple acquisition of equipment such as mechanical milking machines and direct expansion chillers, i. e., this attitude alone is not an appropriate strategy to improve the microbiological quality of milk.

According to Lopes Júnior (2010), the inflammatory response associated with mastitis classifies the pathogens involved as major

pathogens based on a greater increase in SCC (*S. aureus*, *S. agalactiae*, non-*S. agalactiae Streptococcus* spp.) and secondary pathogens based on a lower inflammatory response and smaller variation in SCC (CoNS and *Corynebacterium bovis*). Thus, the variation in SCC estimates the inflammatory response according to the different pathogens of mastitis. In the present study, among the microorganisms analyzed, the genus *Streptococcus* spp. showed the highest correlation with high SCCs. This result agrees with most reports in the literature indicating *Streptococcus* as the cause of elevated SCCs.

Lopes Júnior (2010) also quantified the effect of pathogens causing subclinical mastitis on the variation of SCC. Statistical analysis revealed that, among the pathogens isolated, *S. agalactiae* and CoNS were associated with high and small increases in SCC, respectively, with a significant difference compared to the other pathogens ($P < 0.05$). Souza et al. (2013) evaluated the association between the presence of the contagious pathogens *S. aureus* and *Streptococcus agalactiae* and SCCs in 32 herds. The authors observed a significant association between the isolation of *S. aureus* plus *S. agalactiae* and the SCC of the herd. Herds harboring this combination of pathogens were 9.3 times more likely to have SCCs higher than 400.000 cells/mL when compared to herds showing no isolation. According to Medeiros & Souza (2009), infection with *S. agalactiae* produces high SCCs in individual animals which, in turn, significantly influence the SCC of the herd. The so-called "blitz therapy" is recommended to eradicate *S. agalactiae* from a herd, which consists of the treatment of all infected animals (Medeiros & Souza, 2009).

Taken together, the results of the cited studies and the present findings show major difficulties in meeting the objective limit of 400,000 cells/mL established by the legislation in the case of herds harboring microorganisms of the genus *Streptococcus* spp., especially *S. agalactiae*, and also *S. aureus*.

It is important to understand the influence of the time of year on the occurrence of mastitis. According to Zafalon et al. (2009), the hygiene conditions of a herd are compromised during rainy periods due to the negative influence of elevated humidity levels which, in conjunction with temperature, favor the survival and proliferation of *S. aureus*. Brito et al. (2012[b]) highlighted the existence of variations in the number of mastitis cases according to the season of the year, which increase during the hot and rainy seasons. According to Bueno et al. (2008), the rainy season favors an increase in environmental contamination, the accumulation of mud in the facilities, and the occurrence of dirty teats during milking.

Therefore, the considerable rainfall indices that occurred during the period when the samplings were performed on farms D and E may have contributed to the higher percentage of isolation and higher SCCs observed on these properties. On the other hand, the fact that on farm I the samples were collected during the dry season may explain the finding of the lowest frequency of agents among the nine farms studied and the control of SCCs. However, on farms A, B and C samples were collected during the rainy season and the SCCs of these farms were within the limits of NI-62. The frequency of microorganisms was not high on these farms, in contrast to farms D and E where sample collections also coincided with the rainy period. On farm G, samples were collected during the dry season and this farm exhibited a considerable frequency of microorganisms and the third highest overall mean SCC among the nine farms studied.

Although there is evidence in different studies that predominantly rainy periods can contribute to a higher incidence of mastitis-causing agents, no well-established association between climate and mastitis was observed in the present study. With respect to farms A, B and C where SCCs and the occurrence of contagious agents were controlled, the sampling period coincided with the rainy season. These farms maintained good management practices and had an adequate structure that facilitated cleaning of the facilities and disinfection of the milking equipment. As a consequence, the udders of the animals were constantly free of soil and feces since the areas were fenced and the beds were kept clean. In contrast, various contagious microorganisms were detected on farm G, although samples were collected during the dry season. Thus, the health of a dairy herd can be compromised even in a dry climate when the farm has problems such as deficient milking management. The success of mastitis control thus depends on different procedures that should be incorporated in the farm routine and the occurrence of rain can be related to worsening of animal conditions when microorganisms are present in the environment.

According to the National Mastitis Council (1990), it is important to know that, in addition to climate and season of the year, the size of the herd, type of facilities, nutrition and stressful factors influence the incidence of mastitis. Browye and Edmondson (2010) emphasize that the maintenance of a clean and comfortable environment is essential for the control of mastitis and for quality milk production.

The differences between farms demonstrate that, although located in the same region, they do not

have the same investment, professionals of the same education level, or the same health management and prophylaxis, and therefore differ in terms of mammary gland health and milk quality. According to some authors, an effective approach to reduce somatic cells and bacteria beyond the standards established by the Normative Instruction is through a quality-based milk payment system. However, in addition to a quality payment program, technical assistance programs are essential to teach producers how to improve milk quality. According to Pinheiro (2010), a continuing education program for producers is crucial to improve milk quality and should include actions such as lectures for producers, technical assistance and continuing education projects for producers and employees, manuals and technical information, and providing producers with the results of quality analysis.

CONCLUSION

Some dairy farms encounter difficulties in adapting to the regulations established by NI-62. This study demonstrated a high frequency of samples with a positive CMT, considerable SCCs, and the presence of pathogens due to management and hygiene failures during milking. Microbiological testing revealed *Staphylococcus aureus* as the most common etiological agent of bovine mastitis on the different farms. The genus *Streptococcus* spp. showed the highest coefficient of correlation with elevated SCCs. High rainfall indices contributed to the high rates of isolation and high SCCs.

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