ANTIMICROBIAL RESIDUES IN MILK AND OCCURRENCE OF CLINICAL AND SUBCLINICAL MASTITIS IN HERDS IN THE REGION OF CAMPINAS, SÃO PAULO

[Resíduos de antimicrobianos no leite e ocorrência de mastite clínica e subclínica em rebanhos da região de Campinas, São Paulo]

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ABSTRACT - The aim of this study was to evaluate the occurrence of clinical and subclinical mastitis and antimicrobial residues in milk samples from mammary quarters of 259 lactating cows of five properties in the region of Campinas-SP, Brazil. The Tamis test and the *California Mastitis Test* (CMT) were used to diagnose clinical and subclinical mastitis, respectively. The somatic cells count (SCC) was done in milk samples of weigh jars and cooling tanks. The presence of antimicrobial residues was analyzed in milk samples of the mammary quarters, weigh jars and cooling tanks, with a microbiological test (Delvotest® SP). Milk samples were collected for microbiological isolation of mastitis-causing agents. Clinical and subclinical mastitis occurred in 2.32% to 16.66% and 21.00% to 69.1% of the mammary quarters, respectively. The SCC in the cooling tanks ranged between 221 and 1,124 x 10^3 cells/mL. Coagulase-negative *Staphylococci* were isolated in 45.93% of the milk samples. The analysis of the antimicrobial residues in the weigh jars and in mammary quarters showed 8.33% and 4.30% of samples testing positive, respectively. The presence of antimicrobial residues was detected in 5% of the cooling tanks. The findings point to the need to implement practices that focus on milk quality.

Keywords: somatic cells count, mammary gland, antibiotic therapy.

RESUMO - O objetivo da pesquisa foi avaliar a ocorrência de mastite clínica e subclínica e resíduos de antimicrobianos em amostras de leite de quartos mamárias de 259 vacas em lactação de cinco propriedades da região de Campinas-SP, Brasil. Para o diagnóstico de mastite clínica e subclínica realizou-se testes Tamis e *Califórnia Mastitis Test* (CMT), respectivamente. A contagem de células somáticas (CCS) foi realizada nas amostras de leite dos balões coletores e tanques de resfriamento. A presença de resíduos de antimicrobianos foi avaliada nas amostras de leite dos quartos mamários, balões medidores e tanques de resfriamento através do teste microbiológico (Delvotest® SP). Amostras de leite foram colhidas para isolamento microbiológico dos agentes causadores de mastite. Mastite clínica e subclínica ocorreram entre 2,32% a 16,66% e entre 21,0% a 69,1% dos quartos mamários, respectivamente. As contagens de células somáticas nos tanques de resfriamento variaram entre 221 a 1.124 x 10^3 células/mL. *Staphylococcus* coagulase negativa foram isolados em 45,93% das amostras de leite. A análise de resíduos de antimicrobianos nos balões coletores e quartos mamários revelaram 8,33% e 4,30% de amostras no nível positivo, respectivamente. A análise estatística para frequências de quartos mamários com CMT e níveis de resíduo, somando os escores 1 e 2 de CMT, foi significante ($P < 0,05$). O CMT escore 3 ocorreu em maior porcentagem quando o resíduo foi positivo. Foi detectada a presença de resíduos de antimicrobianos em 5% dos tanques de resfriamento. Os resultados encontrados destacam a necessidade da implantação de políticas concentradas na atenção à qualidade do leite.

Palavras-chave: contagem de células somáticas, glândula mamária, antibioticoterapia.

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INTRODUCTION

In dairy farming, it is extremely important to adopt strategies that maximize production and minimize costs and impacts on the environment. Moreover, it is essential to offer good quality products to consumers. Accordingly, dairy production has been especially careful about effectively treating mastitis and taking efficient measures towards the prevention and control of such condition, in order to ensure sustainable milk production and the good quality of the final product. In the evaluation of milk quality, the following aspects are taken into consideration: somatic cells count, isolation and identification of pathogens and detection of antimicrobial residues (van Schaik et al., 2002).

Antimicrobials are not desirable in milk, as they pose serious risks to human health, mainly because of possible allergic reactions in people who are more sensitive, because of toxic and carcinogenic effects, alterations in intestinal flora and antibiotic-resistant bacteria (Mitchell et al., 1998). Mastitis, which is inflammation of the mammary gland, is responsible for the treatment of lactating cows with antimicrobials. The inflammation of the mammary gland presents several pathological alterations that may influence pharmacokinetics and pharmacodynamics of drugs of intramammary use, applied in the treatment of mastitis (Owens et al., 1997).

Some practices in the dairy farms have been considered as risk factors for the detection of antimicrobial residues in milk. Booth & Harding (1986) conducted studies in English farms, where antibiotic residues were detected in milk. The authors attributed the occurrence of residues to several factors, among which are: failure to observe the withdrawal period for the milk of treated cows, accidental transfer of contaminated milk to the cooling tank, failure to keep treatment record, and non-identification of the treated animals. Besides the mentioned factors, other studies considered different factors to be responsible for the presence of residues in milk: dry cow therapy (Green et al. 2002) and the persistence of residues beyond the withdrawal period, in lactating cows treated with antibiotics (Seymour et al., 1988). Among the agents that cause mastitis, bacteria of the *Staphylococcus* group are significant in the cause of intramammary infections in dairy bovines. Subclinical mastitis caused by *Staphylococcus aureus* is one of the most frequent occurrences, due to its chronic nature and to low rates of cure (Ogola et al., 2007). Inadequate use of antibiotics in the treatment of the disease may compromise its efficiency and may cause the appearance of resistant bacteria (White & Mc Dermott, 2001).

In this context of milk quality and public health, the general purpose of this paper was to evaluate the occurrence of clinical and subclinical mastitis and the presence of antimicrobial residues in the milk of lactating cows, in dairy farms in the state of São Paulo, Brazil.

MATERIAL AND METHODS

Milk samples of 20 cooling tanks, 604 weigh jars and 2,363 mammary quarters of 258 lactating cows were evaluated, in five dairy farms which are part of the same milk tank truck collecting and transportation system, in state of São Paulo, Brazil. The farms were characterized by the letters “A” to “E”, according to milk production and milking procedure (Tab.1). In all dairy farms, every two weeks milk samples were collected from the mammary quarters of lactating cows, from weigh jars and from cooling tanks, for a period of 45 days.

Table 1 - Production characteristics and preventive measures against mastitis in the dairy farms

Characteristics	A	B	C	D	E
Breed	H/Jersey	BS	H / BS	Crossbred	Crossbred
Lactating cows	110	12	69	25	43
Daily production (kg/day)	2,600	95	728	350	150
Number of milking	3	1	2	2	1
Cooling tanks	2	1	1	1	1
Pre-dipping	Yes	No	No	No	No
Post-dipping	Yes	No	Yes	Yes	Yes
Individual paper towel	Yes	No	Yes	No	No

H= Holstein; BS= Brown Swiss

The occurrence of clinical and subclinical mastitis in the five dairy farms was evaluated by examining the mammary quarters of the lactating animals, with the Tamis test and *California Mastitis Test* (CMT), respectively. The mammary quarters that presented clotted milk in the first squirts collected with the Tamis test, and/or visible alterations in the mammary gland (sweeling, heat, redness and pain), were classified as cases of clinical mastitis. Subclinical mastitis was evaluated with CMT and classified in scores 0, 1, 2 e 3 (Daniel et al., 1966). Scores starting from score 1 were considered cases of subclinical mastitis. For somatic cells count (SCC), milk samples were collected from the weigh jars and cooling tanks, in sterilized plastic flasks containing bromothymol preservative. The somatic cells count was done by Somacount *TM* 300 equipment (Bentley Analytical Instruments Inc., Minnesota, USA).

The milk samples were collected from the mammary quarters after washing the teats with soap, drying them with individual paper towels and disinfecting them with swabs containing ethanol solution (70%). Aliquots of milk (10µl) were grown in Petri dishes containing blood agar prepared with 5% (v/v) defibrinated sheep blood, incubated in aerobiosis at 37 degrees for 72 hours. We observed the isolated colonies for morphology, size, pigmentation and the presence of hemolysis. The isolated microorganisms were observed microscopically, with the Gram staining method. Biochemical tests were used for bacterial identification (Winn Jr et al., 2006).

Aliquots of 10µl were grown in Petri dishes containing *Sabouraud* dextrose agar, with Chloramphenicol (100mg.L^{-1}). After the incubation period (3-10 days at 22-25 degrees), colonies of different morphological types were described, for the characterization of filamentous fungi and yeast.

The analyses were conducted with a commercial microbiological test (Delvotest® SP, DSM Foods Specialties B.V., Delft, Netherlands). We considered as positive the tests that did not change from the color purple to yellow. The ampoules that presented a slight change in color were considered to be on the borderline between positive and negative. In order to decrease the occurrence of false positives, due to the interference of natural antimicrobial substances in milk (lysozyme, lactoferrin), milk samples were previously heated in double boiler at 80 degrees for five minutes (Aerts et al., 1995).

The chi-square test (χ^2) and the Kruskal-Wallis test were used for statistical analysis, with the Software Minitab-15 1.3.

RESULTS AND DISCUSSION

Table 2 shows the results of the occurrence of clinical and subclinical mastitis in the mammary quarters of lactating cows, in the dairy farms.

The results indicate that two farms presented indices of clinical mastitis higher than the internationally accepted indices, which should be lower than 3%, according to Reneau (1993).

Table 2 - Relative frequency (%) of the occurrence of clinical and subclinical mastitis in the mammary quarters of lactating cows

Farms	Positive *Tamis*	Negative CMT	Positive CMT
A	4.6	69.2	30.8
B	16.6	30.9	69.1
C	2.8	77.9	22.1
D	2.3	57.0	43.0
E	0.0	83.0	21.0

The variation of the relative frequency of the occurrence of clinical mastitis in the dairy farms is closely related to the characteristics of dairy production and milking procedure. The inflammation process of the mammary gland and the infection level of a herd are due to several factors, among which are: inadequate training of personnel, failure in the milking mechanical system, number of cow lactations, number of milking and type of isolated microorganism (Mc Dermott et al., 1983; Holmes et al., 1996). The dairy farms showed high results of positive reactions to CMT. According to Langoni et al. (2011), the occurrence of subclinical mastitis in Brazilian dairy farms is still high. The results of SCC conducted in the cooling tanks of the dairy farms are shown in Table 3.

The farms presented counts higher than 750,000 cells/mL. Farm B exceeded the count of 1,000,000 cells/mL. This information is troubling, as it exceeds the maximum value established by the Brazilian legislation. Such legislation provides for the maximum limit of 400,000 cells/mL for the area, until the end of the year 2016 (Brazil, 2011).

The correlation between the mean of SCC in the cooling tank and the occurrence of mastitis is high. It varies from 0.50 to 0.96 (Emanuelson & Funke, 1991). High SCC in the tank usually indicates loss in milk production, whereas the maintenance of low SCC in the tank indicates good health of the gland (Schukken et al., 1990). The somatic cells count (SCC) of a cow's milk allows the quantification of the infection level in the mammary gland.

Similarly, periodic evaluation of SCC in the tank milk allows the determination of the mean incidence of mastitis in the herd. Even though we did not find, in this study, significant statistical differences in SCC of the cooling tanks among the dairy farms, the observation by Machado et al.

(2000) corroborate this research, as the farm with the highest rate of clinical and subclinical mastitis presented the highest SCC in the cooling tanks.

Table 4 shows the results of the descriptive analysis of SCC information in the weigh jars of the milk extracted in the milking. There was a significant difference in the SCC in the weigh jars among the farms. Harmon (2001) affirmed that the value up to 200,000 cells/mL is the most recommended for estimating the health of the mammary gland, and that SCC higher than such values suggests inflammatory of infectious processes.

Table 3- SCC in the cooling tanks of the dairy farms

SCC	Farms					
	A1†	A2†	B	C	D	E
Mean	438.25	423.25	693.50	630.00	670.25	680.50
SEM	291.90	157.46	433.67	188.39	143.73	123.07
Minimum value	221.00	297.50	271.00	459.00	546.00	503.00
Maximum value	869.00	645.00	1,124.00	827.00	813.00	784.00
Median	331.50	376.50	689.50	617.00	661.00	717.50

†Farmer with two cooling tanks, SEM. Standard error of means, SCC x 10^3 Kruskall-Wallis P > 0.05

Table 4 - SCC in individual weigh jars in the dairy farms.

Farms	Mean x10^3 cells/mL	Median x10^3 cells/mL	Minimum x10^3 cells/mL	Maximum x10^3 cells/mL	SEM
A	520	163a	3	9,969	1,051
B	1,827	1542b	289	5,684	1,287
C	393	86c	1	6,380	868
D	760	377de	1	4,276	897
E	591	321ae	3	2,881	698

SEM= Standard error means; Means with different superscripts within the same row significantly differ (P > 0.05)

Brito et al. (1999) showed a great variation of SCC and of percentage of mammary quarters with positive CMT, due to types of microorganisms isolated in cases of subclinical mastitis). Furthermore, the value of SCC is also influenced by lactation number, lactation period, season of the year and herd management (Harmon, 1994; Schepers et al., 1997).

Some studies proved that a few simple procedures can contribute to a better development of the dairy system. The importance of the milking procedure, not only during milking, was described by Ott & Novak (2001), in studies on risk factors for the development of mastitis. Their ideas were confirmed in this research. The immersion of teats in a disinfecting solution after milking, the dry cow therapy for all animals, the immediate treatment of clinical cases, and the separation of the animals for milking may contribute to the reduction in the incidence of mastitis (Allore et al., 1998; Berry & Hillerton, 2002).

In this study the most isolated species were *Staphylococcus* (55.75%), *Corynebacterium* (24.60%) and *Streptococcus* (16.73%), followed by yeast (4.74%) and filamentous fungi (2.62%). Included in the *Staphylococcus* species, coagulase-negative *Staphylococcus* accounted for the highest rate of isolations (45.93%), followed by coagulase-positive *Staphylococcus* (28.57%), *Staphylococcus aureus* (17.90%) and *Staphylococcus* spp (7.59%). Such results are closely related to those found in different research that identified the species *Staphylococcus* as the main agent causing mastitis in dairy bovines, whereas coagulase-negative *staphylococcus* were identified as pathogens emerging in some countries (Pyörälä & Taponen, 2009).

Table 5 shows the results of the statistical analysis conducted among different SCC classes of the weigh jars. Such results are associated with the CMT scores obtained in the evaluation of the mammary quarters of the lactating cows. The differences in the CMT occurrences were associated with the SCC classes. The SCC in the weigh jars increased with higher CMT scores. Such result points to the importance of CMT as a supporting tool in the identification of samples with high SCC. The agreement between CMT and SCC found in this study was also found by other authors (Brito et al. 1999; Langoni et al. 2011).

Table 5- SCC classes (%) of the weigh jars in relation to the CMT scores of the mammary quarters in the dairy farms.

SCC classes (x 10^3 cell//mL)	Negative	1	2	3
SCC 1 (< 200)	90.19	88.59	81.87	77.78
SCC 2 (≥ 200 e <400)	3.69	2.66	4.68	3.70
SCC 3 (≥ 400 e <750)	2.23	3.42	5.26	3.17
SCC 4 (≥ 750 e <1,000)	0.97	2.28	1.17	3.17
SCC 5 (≥ 1,000)	2.92	3.04	7.02	12.17

Chi-square test (P<0.05) χ^2=57.667;12DF, ,P=0.000

Table 6 shows the results of milk samples from the cooling tanks, for the presence of antimicrobial residues. Out of all cooling tanks evaluated in the dairy farms, for the presence of antimicrobial residues, 5% were positive in the microbiological test. The milk sample of the cooling tank which was positive in the microbiological test for the detection of antimicrobial residue is related to the dairy farm, which presented high rate of clinical mastitis.

Table 6 - Antimicrobial residues in milk samples from cooling tanks in dairy farms

Cooling tanks	n	%
Negative	19	95
Positive	1	5
Total	20	100

n, number of tanks evaluated

These results corroborate those found by Ruegg & Tabone (2000), who also observed the influence of clinical mastitis in the presence of antimicrobial residues in cooling tanks in dairy farms.

Table 7 shows the results of the analyses for the presence of antimicrobial residues in milk samples of weigh jars of lactating cows in dairy farms. Antimicrobial residues detected in the weigh jars that were on the borderline between positive and negative varied from 0.00% to 1.85%.

Those that tested positive ranged between 0.00% and 8.33%. In only Farm E the milk did not present residues. The cooling tanks in the dairy farms are being supplied with milk contaminated with antimicrobial residues. However, such contamination is not detected with this test in the tanks, due to dilution effect.

Table 7 - Antimicrobial residues in milk samples from weigh jars of lactating cows in the dairy farms

Farm	Negative		Limit		Positive		Total per farm
	n	%	n	%	n	%	
A	205	94.91	1	0.46	10	4.63	216
B	22	91.67	0	0.00	2	8.33	24
C	212	98.15	4	1.85	0	0.00	216
D	94	97.92	0	0.00	2	2.08	96
E	52	100.00	0	0.00	0	0.00	52
Total	585	97.19	5	0.83	14	2.15	604

Table 8 shows the results of analyses for the presence of antimicrobial residues in milk samples of mammary quarters of lactating cows in the dairy farms. Antimicrobial residues were detected in the mammary quarters of the farms. On the borderline between positive and negative, they ranged from 1.05% to 2.15%. Those that tested positive ranged from 0.47% to 4.30%. All farms in evaluation presented antimicrobial residues in milk samples of mammary quarters, testing positive and on the borderline between positive and negative. Thus, the weigh jars are being supplied with contaminated milk.

The distribution of teats in the Delvotest ®SP classes showed that Farm B presented the highest proportion of teats in classes 2 and 3 (6.45% of teats). Farm E presented the lowest proportion (1.92% of teats). However, such differences were not significant in the chi-square test. A bigger sample may have been enough to reveal statistical relevance in this result, as the difference was small (P = 0.051).

Farm B presented significant results in the presence of antimicrobial residues in milk, including the presence of residues in the cooling tank. It also presented higher occurrence of clinical and aspects already dealt with in the literature, including the relation of clinical and subclinical mastitis to the presence of residues in milk (Ruegg & Tabone, 2000; Shaik et al., 2002). subclinical mastitis. Such result emphasizes some

Table 8- Antimicrobial residues in milk samples of mammary quarters of lactating cows in the dairy farms

Farms	Negative		Limit		Positive		Total per farm
	n	%	n	%	n	%	
A	804	96.52	10	1.20	19	2.28	833
B	87	93.55	2	2.15	4	4.30	93
C	831	98.00	13	1.53	4	0.47	848
D	373	97.90	4	1.05	4	1.05	381
E	204	98.08	3	1.44	1	0.48	208
Total	2,299	97.4	32	1.35	32	1.35	2,363

Chi-square test ($P > 0.05$) χ^2 = 9,450.4 DF, P = 0.051

In the dairy farms where there was a good milking procedure (A and C), the animals being treated for clinical mastitis were identified and their milk was discarded. Nevertheless we detected antimicrobial residues in mammary quarters testing positive and on the borderline between positive and negative. In one of these farms (A), despite the animals' identification, there were no treatment records showing the beginning and the end of the treatment, nor the date when the milk was ready for commercialization. This compromises the control of the period necessary for discarding, and it favors the presence of residues in milk. In another farm (C), besides the identification of animals under treatment, there were treatment records showing the dates of the beginning and the end of the treatment and the date when the milk was ready for commercialization, based on the withdrawal period determined by the manufacturer of the medicine applied. However, such records were not available for all milkers. Well-planned pharmacokinetic studies, in order to define the discarding period which is appropriate for the milk, are conducted in the antimicrobials before releasing the milk for commercialization. However, some studies show that, for some drugs, recommendations for withdrawal period were based on limited research information. In order to ensure food safety and avoid antimicrobial residues, veterinarians and farmers must keep detailed records of all treatments with antibiotics supplied in the herd, not only of the mastitis cases (McEwen et al., 1991; Pyörälä, 2002). Another relevant aspect dealt with by other authors refers to the fact that antimicrobial residues occur in milk, on the occasion of dry cow therapy and during the treatment of pre-partum heifers (Mitchell et al., 1998).

Table 9 shows the distribution of antimicrobial residues levels in milk samples from mammary quarters of lactating cows in CMT scores. There was a significant difference ($P < 0.05$) when CMT scores 1 and 2 and residue levels were added. This indicates that the differences in the occurrence of CMT scores are associated with the residue levels. Score 3 CMT occurred in a higher percentage when the antimicrobial residue – analyzed by Delvotest ® SP – tested positive. The results are in line with those observed by other authors in the evaluation of the relation between antibiotic residues and somatic cells count. The higher the SCC, the higher the risks of the occurrence of antimicrobial residues in milk. The authors notice that the presence of antibiotic residues in milk is strongly associated with SCC higher than 400,000 cells/mL (Ruegg & Tabone, 2000). Our study also indicates that the higher occurrences of antimicrobial residues are related to samples that present higher CMT score.

Table 9 - Absolute frequency of CMT scores of mammary quarters and antimicrobial residues in mammary quarters

Residue	CMT				MQ
	0	1	2	3	
1	1,636	285	196	182a	2,299
2	13	5	2	12a	32
3	7	5	3	17b	32
MQ	1,656	295	201	211	2,363

MQ= mammary quarters evaluated; Chi-square test ($P < 0.05$) χ^2 = 117.111,4DF, P = 0.00

CONCLUSIONS

The high incidence of clinical and subclinical mastitis in the mammary quarters of lactating cows, along with the use of uncontrolled drugs, contributed to the presence of antimicrobial residues in mammary quarters, in weigh jars and in cooling tanks. It is necessary to implement public policies for training producers and technicians, and to adopt sanitary measures for milking, in order to minimize compromising the quality of the milk produced, and also to comply with the deadlines determined by the effective legislation.

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