

## Prevalence and etiology of buffalo mastitis and milk somatic cell count in dry and rainy seasons in a buffalo herd from Analândia, São Paulo State, Brazil

[Prevalência e etiologia da mastite bubalina e contagem de células somáticas no leite, nas estações seca e chuvosa, em um rebanho bubalino do município de Analândia, Estado de São Paulo, Brasil]

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### ABSTRACT

The aim of this study was to evaluate somatic cell count (SCC), prevalence and etiology of mastitis in a dairy buffalo herd from Analândia, São Paulo State, Brazil, in the dry and rainy seasons. Additionally, antimicrobial susceptibility profile of microorganisms isolated from milk samples was also evaluated. 1,042 milk samples from female Murrah buffaloes in a dairy farm located in Analândia, São Paulo State, Brazil, collected between May 2011 and November 2012 were analyzed. After the mammary gland physical examination, strip cup test and California Mastitis Test (CMT) were performed. Afterwards, 50mL of milk samples from each mammary quarter were collected aseptically for SCC in automatic equipment and microbiological examination. The antimicrobial sensitivity profile to ampicillin, cefoperazone, ceftiofur, enrofloxacin, gentamicin, neomycin, oxacillin, penicillin, and sulfamethoxazole/trimethoprim was evaluated by disk diffusion method. The monthly average temperature and pluviometric index were obtained from “Centro Integrado de Informações Agrometeorológicas” (CIIAGRO) of “Instituto Agrônomo de Campinas” (IAC). Milk samples with positive results in the microbiological test showed average SCC of 137,720 cells/mL in the dry period and 190,309 cells/mL in the rainy period. Although a higher number of isolated microorganisms was observed in buffalo milk samples during the rainy period (69/600) compared to the dry period (50/442), the season had no significant effect on the frequency of isolation of microorganisms. The main genera of microorganisms isolated were coagulase-negative *Staphylococcus* (38.4%), *Streptococcus agalactiae* (28.8%), and *Bacillus* spp. (7.56%) during the dry season and *Corynebacterium* sp. (23.5%), *Streptococcus* spp. (32.3%), and *Streptococcus agalactiae* (9.24%) during the rainy period. Multidrug resistance was observed in 30.1% of the isolated microorganisms.

Keywords: antibiogram, *Bubalus bubalis*, SCC, mammary gland, microorganisms

### RESUMO

O objetivo do presente estudo foi avaliar a contagem de células somáticas, a prevalência e a etiologia da mastite bubalina nas estações seca e chuvosa em um rebanho de bubalinos do município de Analândia, estado de São Paulo, Brasil. Adicionalmente, verificou-se o perfil de sensibilidade antimicrobiana dos micro-organismos isolados nas amostras de leite das búfalas. Foram avaliadas 1.042 amostras de leite de búfalas da raça Murrah pertencentes a uma propriedade rural localizada no município de Analândia-SP, obtidas no período de maio de 2011 e novembro de 2012. Após o exame físico da glândula mamária, foram realizados o teste da caneca de fundo escuro e o California Mastitis Test (CMT); em seguida, foram

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colhidas, de forma asséptica, amostras de 50mL de leite de cada quarto mamário, para a contagem de células somáticas (CCS) em aparelho automático e exame microbiológico. Também, foi avaliado o perfil de sensibilidade antimicrobiana a ampicilina, cefoperazona, ceftiofur, enrofloxacina, gentamicina, neomicina, oxacilina, penicilina e sulfametoxazol/trimetoprim, pelo método de difusão em disco. A temperatura média e o índice pluviométrico mensais foram obtidos no Centro Integrado de Informações Agrometeorológicas (CIIAGRO) do Instituto Agronômico de Campinas (IAC). Notou-se que as amostras de leite com resultado positivo no exame microbiológico apresentaram CCS média de 137.720 células/mL, no período seco, e 190.309 células/mL, no período chuvoso. Embora tenha se constatado maior índice de isolamentos de micro-organismos nas amostras de leite obtidas no período chuvoso (69/600) do que no período seco (50/442), a frequência de isolamentos não foi influenciada significativamente pela estação do ano. Os principais gêneros de micro-organismos isolados durante o período seco foram *Staphylococcus coagulase negativa* (38,4%), *Streptococcus agalactiae* (28,8%) e *Bacillus spp.* (7,56%), e no período chuvoso *Corynebacterium sp.* (23,5%), *Streptococcus spp.* (32,3%) e *Streptococcus agalactiae* (9,24%). Verificou-se resistência a três ou mais antimicrobianos em 30,1% dos micro-organismos isolados.

*Palavras-chave:* antibiograma, *Bubalus bubalis*, CCS, glândula mamária, micro-organismos

## INTRODUCTION

The Brazilian buffalo herd in 2011 was 1.3 million animals and São Paulo State had the sixth largest herd of the country with 75,700 buffaloes (5.9%) (Instituto..., 2012). The Brazilian production of buffalo milk and its derivatives has been growing annually, particularly in Southeast region, due to the appreciation of the physico-chemical characteristics of buffalo milk (Madella-Oliveira *et al.*, 2005).

The buffaloes, as well as bovines, may present mastitis, resulting in decreased milk production, changes in concentration of milk components and increase in somatic cell count (SCC), which varies according to the intensity and length of inflammation (Hamza and Choudhuri, 1994). Similarly to cows, the most frequently isolated bacteria in buffaloes mastitis are *Staphylococcus* and *Streptococcus* genera (Bastos and Birgel, 2011; Medeiros *et al.*, 2011; Silva *et al.*, 2011), however, the habit of buffaloes to enter marshes can favor mammary infection by environmental microorganisms, particularly fungi, algae and enterobacteria (Osman *et al.*, 2009).

Studies also report seasonal influence in isolation of microorganisms which cause mastitis in cows in Brazil (Ferreira *et al.*, 2006; Zafalon *et al.*, 2008) and buffaloes in Turkey (Özenç *et al.*, 2008; Gürler *et al.*, 2013).

The aim of this study was to evaluate SCC, prevalence and etiology of mastitis in dairy buffaloes in dry and rainy seasons in a herd

raised in Analândia, São Paulo State, Brazil. Additionally, the antimicrobial susceptibility profile of microorganisms isolated from milk samples was evaluated.

## MATERIAL AND METHODS

1,042 milk samples from primiparous and pluriparous female Murrah buffaloes, submitted to mechanic milking once a day, from a rural property located in Analândia, São Paulo State, Brazil, collected from May 2011 to November 2012 were evaluated.

After the mammary gland physical examination (Radostitis *et al.*, 2007), the milk from each mammary quarter was submitted to the strip cup test and California Mastitis Test (CMT) (Schalm and Noorlander, 1957). For somatic cell count (SCC) and microbiological test, 50mL of milk samples of each mammary quarter were collected aseptically in sterilized plastic bottles with and without bronopol, respectively. SCC was obtained in automatic equipment (Somacount 300) by flow cytometry utilizing bovine matrix stands according to International Daily Federation (International..., 2006). Microbiological test was performed with standard techniques of NMC protocols (Laboratory..., 1999) by streaking 10uL of each milk sample in 5% sheep blood agar and MacConkey agar, maintained at 37°C in aerobic conditions, with daily readings for 72 hours. Simultaneously, all milk samples were cultured on Sabouraud agar with dextrose and maintained at 37°C to isolate and identify fungi. The

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microorganisms were identified according to morpho-tinctorial, biochemical and culture characteristics (Quinn *et al.*, 2011). Additionally, the antimicrobial sensitivity profile to ampicillin (10µg), cefoperazone (75µg), ceftiofur (30µg), enrofloxacin (5µg), gentamicin (10µg), neomycin (30µg), oxacillin (5µg), penicillin (10IU) and sulfamethoxazole/trimethoprim (25 µg) was evaluated by the disk diffusion method (Clinical..., 2006).

The monthly average temperature and pluviometric index were obtained from "Centro Integrado de Informações Agrometeorológicas" (CIIAGRO) of "Instituto Agronômico de Campinas" (IAC). As an adaptation from Zafalon *et al.*, (2010) the months of the year were classified as "dry" (average rainfall of approximately 30mm and maximum <60mm) and "rainy" (average rainfall of approximately 120 mm and minimum >70mm).

The average temperatures and rainfall values in dry and rainy periods were submitted to analysis of variance (ANOVA) and Student t test to compare pairs of means (P<0.05). To assess the influence of dry and rainy periods in mastitis occurrence the chi-square test was used. To

evaluate SCC obtained both in dry and rainy periods, the nonparametric Kruskal-Wallis test followed by Dunn's posttest comparison were used (Triola, 2008).

The study was approved by the Ethics Committee on Animal Use (Comissão de Ética no Uso de Animais - CEUA) of Faculdade de Ciências Agrárias e Veterinárias - FCAV/UNESP/Jaboticabal Campus (Protocol No. 017093/12).

## RESULTS AND DISCUSSION

According to the pluviometric index in the region of Analândia-SP during the study period, seven months were classified as dry and 12 months as rainy. The lowest rainfall (0.1mm) was observed in July 2011, while the highest (266.1mm) was in January 2012. The lowest monthly minimum temperature (1.5°C) was registered in June 2011 and the highest monthly maximum temperature (30.9°C) was registered in October 2012 (Table 1).

A significant difference (P<0.05) was observed both for average temperature and rainfall in dry and rainy seasons (Table 2).

Table 1. Meteorological data from Analândia-SP region and monthly classification in dry and rainy, from May 2011 to November 2012

Month/year	Minimum temperature (°C)*	Maximum temperature (°C)*	Average temperature (°C)*	Rainfall (mm)*	Classification
May/2011	8.0	24.7	13.4	5.5	Dry
June/2011	1.5	24.0	11.3	42	Dry
July/2011	6.2	25.7	13.5	0.1	Dry
August/2011	3.6	27.7	15.0	11.6	Dry
September/2011	8.1	29.5	15.0	13.0	Dry
October/2011	12.1	27.9	17.3	141.4	Rainy
November/2011	10.9	28.1	16.4	142.2	Rainy
December/2011	13.6	28.9	17.9	221.1	Rainy
January/2012	15.1	27.0	18.0	266.1	Rainy
February/2012	18.0	30.5	19.6	97.7	Rainy
March/2012	15.0	29.2	18.4	79.3	Rainy
April/2012	14.8	27.9	17.9	147.5	Rainy
May/2012	10.0	23.9	13.8	103.1	Rainy
June/2012	11.2	23.2	14.5	190.3	Rainy
July/2012	5.2	24.5	12.4	42.7	Dry
August/2012	9.3	26.2	14.1	2.4	Dry
September/2012	7.4	28.9	15.2	77.4	Rainy
October/2012	13.5	30.9	18.8	86.7	Rainy
November/2012	13.5	28.9	17.7	130.1	Rainy

\*Source: CIIAGRO

Table 2. Average temperature and rainfall of Analândia-SP region, from May 2011 to November 2012, according to the time of the year (dry and rainy)

Period	Temperature (°C)	Rainfall (mm)
Dry	19.8A	16.8A
Rainy	22.5B	140.2B

Means in the same column followed by the same letter do not differ by Student t test ( $P>0.05$ ).

Regarding the CMT, of the 442 samples obtained in the dry period, 27 (6.11%) had positive reactions and 415 (93.9%) were negative to the test, while of the 600 samples collected during the rainy season, 60 (10.0%) had positive results and 540 (90.0%) were negative to the test (Table 3). During physical examination and strip cup test no changes were observed in mammary glands or in milk characteristics, respectively.

Table 3. Absolute and relative values of CMT from buffalo milk samples, from May 2011 to November 2012, in dry and rainy periods

Period	No. of samples	CMT Result			
		-	+	++	+++
Dry	442	415 (93.9%)	11 (2.49%)	9 (2.04%)	7 (1.58%)
Rainy	600	540 (90.0%)	17 (2.83%)	13 (2.17%)	30 (5.00%)
Total	1,042	955 (91.7%)	28 (2.69%)	22 (2.11%)	37 (3.55%)

-: negative reaction; +: slight reaction; ++: moderate reaction; +++: intense reaction

According to Brito *et al.* (1997), positive reaction in CMT generally indicates inflammatory reaction in the mammary gland, suggesting subclinical mastitis. However, other factors of non-infectious origin, such as lactation stage, animal age and season, among others, may also influence CMT results, which is considered an indirect indicator of milk SCC, but the effects are minimal in uninfected mammary glands (Reneau, 1986; Harmon, 1994). Buffaloes are considered less susceptible to mastitis than cows, because of specific anatomical and physiological characteristics; several studies have reported low occurrence of clinical and subclinical mastitis in this species, in spite of the similarity of the etiologic agents in the two species (Jorge *et al.*, 2005; Kapronezai *et al.*, 2005; Carvalho *et al.*, 2007; Sollecito *et al.*, 2011).

Regardless of CMT results, all the 1,042 samples were submitted to the microbiological test and

119 (11.4%) of them presented bacterial growth (Table 4). Although a greater number of isolated microorganisms was observed in milk samples obtained during the rainy period (69) than in dry one (50), the season had no significant influence on the frequency of isolation of microorganisms (Table 4). The greater number of isolations during the rainy period may be related to higher temperature and humidity (Table 2), which favors the proliferation of mastitis-causing microorganisms (Ferreira *et al.*, 2006; Zafalon *et al.*, 2008; Andrade *et al.*, 2011). In addition, only 4.00% (2/50) and 13.0% (9/69) of milk samples with microbiological isolation in dry and rainy periods, respectively, showed slight to intense reaction in CMT, in accordance with the statement of Kapronezai *et al.* (2005) that there is high frequency of asymptomatic carriers in the herd or that CMT is not a good screening test for mastitis in buffaloes, due to the occurrence of false-positive and false-negative results.

Table 4. Absolute and relative values obtained in microbiological test from buffalo milk samples, from May 2011 to November 2012, in dry and rainy periods

Period	No. of samples	Microbiological test result	
		Negative	Positive
Dry	442	392A (88.7%)	50A (11.3%)
Rainy	600	531A (88.5%)	69A (11.5%)
Total	1,042	923 (88.6%)	119 (11.4%)

Means in the same column followed by the same letter do not differ by chi-square test ( $P>0.05$ ).

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The main isolated microorganisms during the dry period were coagulase-negative *Staphylococcus* (*Staphylococcus* spp.) (38.4%), *Streptococcus agalactiae* (28.8%) and *Bacillus* spp. (7.56%) and in the rainy period were *Corynebacterium* sp. (23.5%), *Streptococcus* spp. (32.3%) and *Streptococcus agalactiae* (9.24%) (Table 5). Other studies also reported *Staphylococcus* spp., *Streptococcus* spp., *Corynebacterium* sp. and Gram-negative bacteria as the most isolated etiologic agents from buffalo milk samples

of different breeds (Bastos and Birgel, 2011; Medeiros *et al.*, 2011; Silva *et al.*, 2011).  $\beta$ -hemolytic streptococcus (*Streptococcus agalactiae*) are more pathogenic to domestic ruminants (Laboratory..., 1999; Quinn *et al.*, 2011). The presence of hemolysis ( $\alpha$ -hemolysis) should be considered an additional virulence factor, since hemolytic strains can release the iron ion from red blood cells; which is an essential element for bacterial growth (Laboratory..., 1999; Quinn *et al.*, 2011).

Table 5. Absolute and relative values of isolated microorganisms, from May 2011 to November 2012, according to dry and rainy periods

Microorganism	Period		Total
	Dry	Rainy	
<i>Bacillus</i> spp.	9 (7.56%)	0 (0.00%)	9 (7.56%)
<i>Corynebacterium</i> sp.	0 (0.00%)	28 (23.5%)	28 (23.5%)
<i>Klebsiella pneumoniae</i>	1 (0.84%)	0 (0.00%)	1 (0.84%)
<i>Pasteurella multocida</i>	1 (0.84%)	0 (0.00%)	1 (0.84%)
<i>Staphylococcus aureus</i>	1 (0.84%)	0 (0.00%)	1 (0.84%)
<i>Staphylococcus</i> spp.	19 (16.0%)	5 (4.20%)	24 (20.2%)
<i>Streptococcus agalactiae</i>	15 (12.6%)	11 (9.24%)	26 (21.8%)
<i>Streptococcus dysgalactiae</i>	2 (1.68%)	5 (4.20%)	7 (5.88%)
<i>Streptococcus</i> spp.	0 (0.00%)	16 (13.4%)	16 (13.4%)
<i>Streptococcus</i> $\alpha$ -hemolytic	1 (0.84%)	0 (0.00%)	1 (1.68%)
<i>Streptococcus</i> $\beta$ -hemolytic	0 (0.00%)	2 (1.68%)	2 (1.68%)
<i>Staphylococcus</i> spp + <i>Staphylococcus</i> $\beta$ -hemolytic	0 (0.00%)	1 (0.84%)	1 (0.84%)
<i>Staphylococcus</i> spp + <i>Streptococcus dysgalactiae</i>	1 (0.84%)	0 (0.00%)	1 (0.84%)
<i>Staphylococcus</i> $\beta$ -hemolytic + <i>Streptococcus</i> $\alpha$ -hemolytic	0 (0.00%)	1 (0.84%)	1 (0.84%)
Total	50 (42.0%)	69 (58.0%)	119 (100%)

Regarding the SCC, milk samples with negative results in microbiological test showed average values of 99,513 cells/mL and 125,606 cells/mL in dry and rainy periods, respectively. On the other hand, milk samples with positive results in microbiological test presented average values of 137,720 cells/mL and 190,309 cells/mL in dry

and rainy seasons, respectively (Table 6). The variations in the SCC of milk samples with positive results in microbiological test collected during dry and rainy periods may be related to the stage of infection and the type of the isolated microorganism (Souza *et al.*, 2009; Medeiros *et al.*, 2011; Sollecito *et al.*, 2011).

Table 6. Means and standard deviations of SCC of Murrah buffalo milk samples, according to the results of microbiological test and period of the year

Period	SCC (cells/mL)	
	Negative microbiological test	Positive microbiological test
Dry	99,513±243,887Aa	137,720±623,118Aa
Rainy	125,606±421,243Aa	190,309±560,079Ab

Means in the same column followed by the same capital letters and same lower case letters in the same line do not differ by Dunn's test (P>0.05).

Regardless of the period of the year, the antibiogram results showed that 94.0% of the isolated bacteria were sensitive to gentamicin, 88.0% to ceftiofur, 88.0% to

cefoperazone, 84.3% to ampicillin, 78.3% to oxacillin, 74.7% to penicillin, 71.1% to enrofloxacin, 67.5% to neomycin and 60.2% to sulfamethoxazole/trimethoprim (Figure 1). The

same test indicated that 39.8% of the isolated bacteria were resistant to sulfamethoxazole/trimethoprim, 32.5% to neomycin, 28.9% to enrofloxacin, 25.3% to penicillin, 21.7% to oxacillin, 15.7% to ampicillin, 12.0% to ceftiofur, 12.0% to cefoperazone and 6.00% to gentamicin (Figure 1). Multiresistance, defined as resistance to three or more types of antibiotics tested (Schwarz *et al.*, 2010), was found in 30.1% of the isolated bacteria, of which 4.00% were resistant to three antibiotics, 16.0% to four

antibiotics and 76.0% were resistant to five or more antibiotics, simultaneously. The high percentage of resistance to penicillin, neomycin and sulfamethoxazole/trimethoprim and the presence of multidrug-resistant strains may be related to the indiscriminate use and dose, the range of application and duration of the antimicrobial use, allowing the selection of resistant strains, leading to a serious problem of public health.

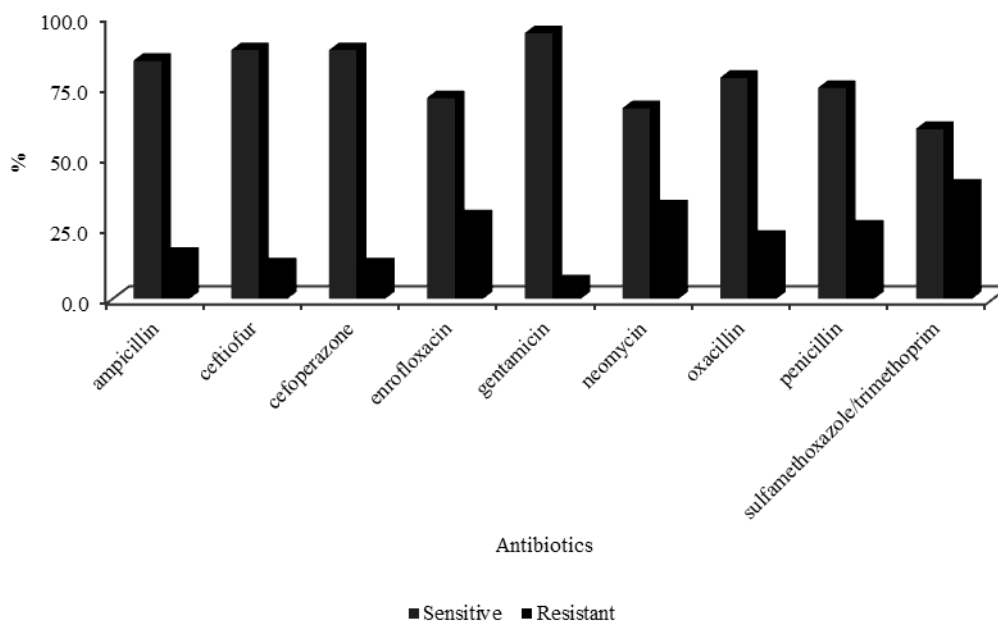


Figure 1. Antimicrobial sensitivity and resistance profiles of microorganisms isolated from Murrah buffalo milk samples.

### CONCLUSION

Buffalo milk samples with positive result in microbiological test showed average SCC of 137,720 cells/mL in dry period and 190,309 cells/mL in rainy period. No influence was observed regarding the dry and rainy seasons on the frequency of isolation of microorganisms. The main microorganisms isolated in the dry period were coagulase-negative *Staphylococcus*, *Streptococcus agalactiae* and *Bacillus* spp. and in the rainy period were *Corynebacterium* sp., *Streptococcus* spp. and *Streptococcus agalactiae*. Multidrug resistance to three or more drugs was observed in 30.1% of the isolated microorganisms.

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