

Bovine Mastitis in the Metropolitan Area of Curitiba: Antibiotic Resistance and Antimicrobial Control of the Infection

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ABSTRACT

A study from cows with mastitis was performed and Staphylococcus aureus was the predominant pathogen in 46.4 % among 153 studied strains from 276 milk samples of infected cows. Antibiotic resistance of 71 S. aureus isolates was determined in order to search resistant strains to antibiotics of clinical interest, as well as to determine their degree of multi-resistance. It was found that 60% of the S. aureus strains presented resistance to β -lactams, but none to oxacillin, teicoplanin or vancomycin. On the other hand, with the aim of reducing the use of current antibiotics and their associated resistance, a new formulation was introduced. The antimicrobial compounds (P22-P32), demonstrated to be effective in 55% of the 76 mastitis cases studied. The use of P22-P32 reduced the number of somatic cell to less than 300,000 SCC/mL⁻¹ in 75.2 % of milk samples analyzed, normalizing the milk quality, fat and lactose levels and increasing the volume of production in 10.1 %.

Key words: mastitis, *Staphylococcus aureus*, antibiotic resistance, infection reduction

INTRODUCTION

Mastitis is considered an inflammatory reaction of the mammary gland and this may be caused by infectious, traumatic and/or toxic agents (Bramley et al., 1996).

The infection of the mammary gland results in the reduction of milk production and changes in the chemical composition, depending on the intensity and state of the disease (Compton et al., 2007, a). Mastitis is manifested as clinic and sub-clinic forms.

The incidence of the latter is more frequent in cows than the clinical disease. Sub-clinic mastitis does not show evident changes in the udder and in the aspect of the milk; however it has a great impact on quality and productivity parameters (Hagnestam and Emanuelson, 2007).

Different microorganisms have been isolated from the bovine mastitis. *Staphylococcus aureus* (Ferreira et al., 2006; Anderson et al, 2006), *Streptococcus agalactiae*, and *Streptococcus dysgalactiae* are among the most frequent and prevalent microorganisms, partly due to the

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contagious mode of transmission. *S. aureus* is able to produce a variety of extra cellular toxins and virulence factors in the host (Swinkles et al., 2005; Compton et al. 2007 a e b).

Other microorganisms such as yeasts are frequently associated with bacteria, forming biofilms that may increase the adherence and invasion of pathogenic *S. aureus*, impairing the effective action of antibiotics (Hensen, et al. 2000, Gonçalves et al., 2010).

This microbial infection produces the invasion of the mammary gland and it is followed by an increase in the number of somatic cells and leukocytes in the milk, reducing its quality (Burvenich et al., 1995; Dettleux, 2004).

In order to control the effects and incidence of mastitis, different kinds of antibiotics of current use in human medicine are employed, and as a consequence of antibiotic pressure, strains with chromosomal or extra-chromosomal resistance have appeared (Mendes et al., 2005, Kato et al., 2008, Swinkles et al., 2005).

At present, β -lactam antibiotics, particularly penicillins and cephalosporins, are being intensively used in humans and in animals. There has been emergence of antibiotic multiresistant strains impacting the public health (Huang, 2006). Susceptibility tests are of utmost importance and used to determine the resistance and to select the most convenient antibiotics to be employed (Nader et al. 2007).

On the other hand, non-classic antibiotic type of compounds were introduced as food bioprotectors and showed effective action on *S. aureus* and other microorganisms.

They are produced by microbial fermentation (Ryan et al., 1998; Parada et al., 2007). The aim of this work was to study the antibiotic resistance of *S. aureus* and develop a new formula with bioactive antimicrobials for mastitis medication, in order to offer new alternatives to the use of common medical antibiotics and to reduce the associated bacterial resistance.

MATERIAL AND METHODS

Sampling and clinical observation

The present study was carried out in 12 dairy farms located in the metropolitan region of Curitiba. Milk samples (276) were taken aseptically from the infected quarters of cows,

before and after the medication and were transported under refrigeration for analysis.

Udder consistence and symmetry, milk smelling and physic aspects were previously observed.

Diagnostic of Mastitis

Milk quality and the diagnostic of mastitis was evaluated by the microbiological examination, indirect physico-chemical methods and by the California Mastitis Test and Somatic Cells Count (SCC) as reported by Fernandes, (2006 a, b); Galiero and Morena, (2000) and Schalm and Noorlander (1957). Milk samples from cases with suspected mastitis were tested by the California Mastitis Test (Fatec). Somatic Cell were counted using flux citometry in a Somacount 300 (Bentley Instruments) according to International IDF Standard 148-2 (1991).

Isolation and microbial characterization

Isolation and bacterial identification were carried out after plating the diluted milk samples (0.1 mL) on blood agar plates and incubation at 37°C for 24 h.

Preliminary identification of independent isolates was made by colony morphology, formation of hemolytic halos and by microscopy, observation. Presumptive strains of *S. aureus* were plated on Mannitol Salt Agar and Baird Parker Agar containing egg-yolk and telluride. Biochemical identification was made by using API gallery (Biomérieux), catalase and coagulase tests (Koneman et al., 2001).

Yeasts were detected on Potato Dextrose Agar incubated at 30°C, and by microscopic observation.

All the strains were maintained at - 80°C in Trypticase Soy Broth containing 40.0 % glycerol.

Antimicrobial Susceptibility Tests

The antibiotic susceptibility tests were done by the Agar Disc Diffusion method, MIC₅₀ and MIC₉₀ according the Clinical Laboratory Standards Guide (CLSI, 2008). For quality control, strains of *S. aureus* ATCC 25923 and ATCC 29213 were used as reference in all assays. *S. aureus* strains were grown on Trypticase Soy Agar (TSA) 24 hours at 37°C, and then used to prepare the respective inocula. Cells were suspended in saline solution of NaCl 0.9%. Turbidity was adjusted to 0.5 McFarland standard (about 10⁸ UFC/mL), and used as inoculum for the antibiotic susceptibility tests (Koneman et al., 2001).

Resistance was evaluated by usual criteria (Bauer et al., 1966; Rossi and Andreazzi, 2005; CLSI, 2008). Disks from Newprov Laboratory, Pinhais-PR, Brazil containing the following antibiotics were used: amoxicillin 10 µg; penicillin 10 µg; oxacillin 10 µg; cephalexin 30 µg; cephoxitin 30 µg; cloranphenicol 30 µg; erythromycin 15 µg; gentamicin 10 µg; teicoplanin 30 µg and vancomycin 30 µg. Oxacillin was used for detection of methicillin-resistant strains, since it is more stable and provides reliable results (Huang et al., 2006).

Therapeutic protocol for mastitis treatment

A group of 76 cows with subclinical and clinical mastitis including, some chronic mastitis with high somatic cells counts was medicated with P22-P32 compounds usually produced by microbial fermentation and generally accepted as safe and not included in the group of antibiotics used in medicine. The formulation contained 500 mg of P22 (antimicotic) and 250 mg P32 (antibacterial) dissolved in 10.0 mL of sterile solution of glycerol-water as vehicle. Daily intramammary application was made during three consecutive days. As control group, 76 normal cows were injected with the placebo, glycerol/water without the active compounds. Milk samples from both groups were collected before application of P22-P32 formulation, as well as after 8, 20, 30 and 180 days and used for control analysis.

Data of were expressed as the average, with $p < 0.05$, values obtained using the geoR Statistic Package, LEG, UFPR (Ribeiro et al., 2006).

Depuration of P22-P32 in milk

Presence of antimicrobial P22 in milk was determined after milk coagulation by trichloroacetic acid 10 %, and then centrifugation (Nascimento et al. 2006). The concentration of the agent was measured in the supernatants by spectrophotometry at 320 nm, using the original compound as control. At the end of the therapeutic

treatment, confirmation of absence was made by HPLC instrument (Shimadzu-SINC) according to Food and Drug Administration (2001). On the other hand, presumptive test for detection of P32 compound, as well as for the association of both active compounds were carried out by incorporation in sterile milk, using *Lactobacillus* and *Streptococcus* as tester strains and incubation at 37°C. Normal acidification and coagulation after 12-24 hours was an indication of absence of antimicrobial activity (Germano et al., 2003).

Protein, fat and lactose analysis

Protein, fat and lactose concentration were determined by Infrared Absorption lecture in a Bentley 2000 instrument.

These analyses were carried out in the APCBRH Laboratory. Determination of contents was made as recommended by the International Dairy Federation Standards (1996).

RESULTS AND DISCUSSION

The microorganisms usually found in bovine mastitis are Gram positive and Gram negative bacteria, and some times yeasts. Among the Gram positive bacteria, *Staphylococcus aureus* is the most frequent contaminant in the milk udder of cows with mastitis (Gonçalves and Kozicki, 1997; Huang et al., 2006).

In the present study *S. aureus* was found to be predominant in 46.4% of the cases and the percentage of *Micrococcus* and other type of microorganisms are reported in Table 1.

Encapsulation of *S. aureus* within biofilms could protect them against the action of usual antimicrobial agents (Hensen et al., 2000).

This fact suggests eventual association of bacteria and yeasts producing internal biofilms, increasing the bacterial adherence into the udder. In our case 7.8 % of the samples contained yeasts.

Table 1 - Microorganisms isolated from milk.

Microorganism	Isolates	%
<i>Staphylococcus aureus</i>	71	46.4
<i>Micrococcus</i>	36	23.5
<i>Streptococcus sp.</i>	6	3.9
Gram positive cocci	10	6.5
Gram positive bacillus	14	9.2
Gram negative bacillus	4	2.6
Yeasts	12	7.8

Resistance of *S. aureus* to antibiotics

The intensive use of antibiotics in medicine as well as growth promoters in feed induce selective pressure and resistance in the microorganism, causing epidemic dissemination and difficulties in the cure of the infection (Rossi and Andreazzi, 2005). The isolation of *S. aureus* resistant to β -lactams, cefens, aminoglycosides, lincosamides

and macrolides is frequent (Rossi and Andreazzi, 2005; Luthje and Schwarz, 2006; Matos et al., 2007; Nader et al., 2007). In the present research antibiotic resistance was also studied, and it was observed that most bovine strains were susceptible to various antibiotics with the exception of β -lactams, that present a high degree of resistance as is shown in Table 2.

Table 2 - Antibiotic susceptibility of *S. aureus*

Antibiotic	n			Resistant (%)
	S	I	R	
Amoxicillin	29	0	42	59.1
Penicillin	24	0	47	66.2
Oxacillin	71	0	0	0
Cephalexin	57	3	11	15.5
Cephoxitin	57	5	9	12.5
Chloranphenicol	61	10	0	0.0
Erythromycin	31	34	6	8.5
Gentamicin	54	13	4	5.6
Teicoplanin	71	0	0	0.0
Vancomycin	71	0	0	0.0

[†]According to CLSI (2008) standards; (n=71).

S=susceptible; I= intermediary; R=resistant.

Therapeutic Effects and reduction of SCC

After intramammary application of P22-P32 suspension, drainage and reduction of undesirable microorganisms and inflammatory material from infected udder was observed.

The product had a revulsive effect, promoting the elimination of infectious clumps, necrotic tissues and exudates from the udder after eight to twelve hours post-application.

It was observed that the edema, redness and sensibility disappeared in the following two days.

The control of Somatic Cells Counts in bovine mastitis is a useful parameter to evaluate the

evolution of the disease.

During experimental trials it was observed clinical improvement of the medicated group. The medication with P22-P32 formulation was effective in a high percentage of the mastitis cases (55%), reducing significantly the SCC as shown in Fig.1.

Placebo did not cause secondary reactions in the control group.

The P22-P32 formulation was tested after 3 months at 25°C, and it was demonstrated antimicrobial stability and normal biological activity.

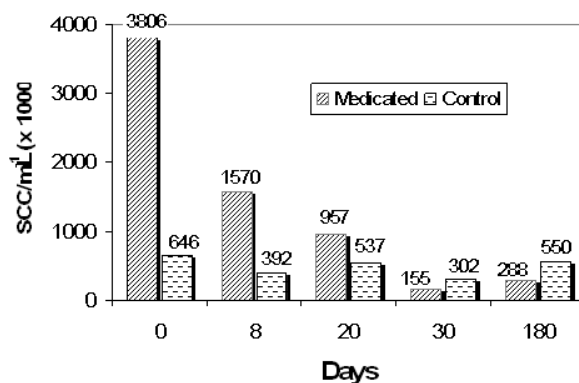


Figure 1 – SCC/mL⁻¹ (x 1000) in medicated and control groups before medication and at 8, 20, 30, 180 days after medication, p< 0,01 (n= 152).

Depuration of P22-P32 after medication

It is known that milk with antibiotics is not permitted by the official organisms of control (Anvisa, 2003).

Depuration of P22 agent in milk during and after the medication was made log Spectrophotometric Method at 320 nm, (DSM Food Specialties, 2001) using as reference standard the original substance. The concentration of P22 compound in milk after application of the third dose decayed gradually, and the agent disappeared completely after 24 h and this fact was confirmed in HPLC determinations. The result is shown in Fig 2. In usual antibiotics, this period of depuration is usually longer (Rossi et al., 2005). Presence of P32 alone and in association (P22-32) was carried out by inhibition of milk fermentation, using

Lactobacillus and *Streptococcus* as sensitive strains.

Milk coagulation and low pH observed after 24 hours, is indicative of the absence of antimicrobial agents against Gram positive bacteria.

After one day post-medication the milk was apt for safe use and within the Brazilian and international norms. It is worthy to mention that P22 substance is recognized as GRAS.

Treatments with other antibiotics produce longer lack periods and remain in milk for larger periods of time to reinitiate a safe milking 72-96 hours (Oliver et al., 2004 a, b; Rossi et al., 2005). In our study with P22-P32 this period was reduced to only 24-48 hours, with the concomitant hygienic and economical benefits

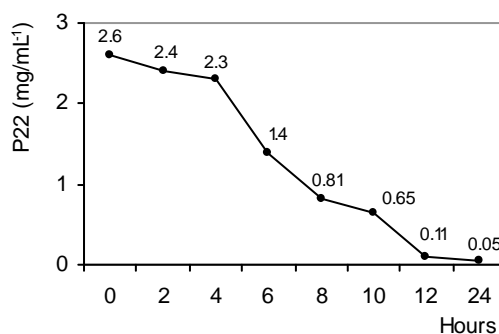


Figure 2 – Depuration of P22 in milk.

Improvements in milk quality

In mastitis infectious disease, cows reduced the milk volume per day and produce alterations in chemical composition lowering the lactose and fat content (Bramley et al., 1996, Ribas et al., 2004). The medicated group under study 76 milk samples

was observed during 180 days.

The mean of lactose concentration increased during the first 30 days up to normality, while protein content did not have significant variations, as can be observed in Fig. 3.

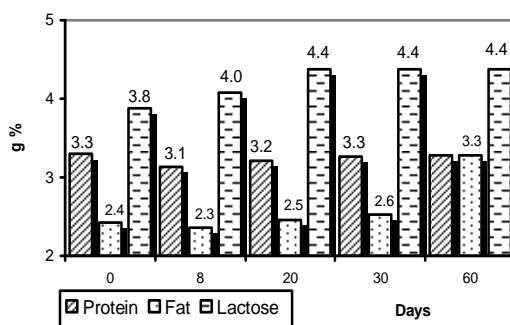


Figure 3 - Evolution of protein, fat and lactose in the medicated group (n= 76); $p < 0.05$; Standard Deviations were 0.34 for protein, 0.35 for lactose and 0.77 for 4fat.

Lipids mean concentration showed a gradual but no significant increase throughout the first 30 days and normalizing at 60 days (Ribas et al., 2004).

Average of Milk production in the medicated group at the end of the experiment had an increment in productivity of 10.1 % with respect to the control group. The mean value increased from 28.7 L/cow/day to 31.2 L/cow, indicating good recovery of the udder function.

P22-P32 formulation reduced in 75.2 % the udder infections in clinical and subclinical cases of mastitis, improving lactose and milk production.

Since the use of antibiotics is desirable to be restricted in treatment of mastitis due to the emergence of antibiotic resistance.

These results indicated that the product may be an alternative control of udder infections, contributing to animal and public health. Further trials with more animals will be carried out in the near future.

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