NaOCl effect on biofilm produced by *Staphylococcus aureus* isolated from the milking environment and mastitis infected cows[1]

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ABSTRACT.- Melo P.C., Sousa C., Botelho C., Oliveira R. & Nader-Filho A. 2014. **NaOCl effect on *Staphylococcus aureus* biofilm isolated from the milking environment and mastitis infected cows.** *Pesquisa Veterinária Brasileira 34(2):109-113.* Departamento de Ciências Agrárias e Ambientais, Universidade Estadual de Santa Cruz, Campus Soane Nazaré de Andrade, Rodovia Jorge Amado, Km 16, Salobrinho, Ihéus, BA 45662-900, Brasil. E-mail: policame@yahoo.com.br

Biofilms constitute a physical barrier, protecting the encased bacteria from detergents and sanitizers. The objective of this work was to analyze the effectiveness of sodium hypochlorite (NaOCl) against strains of *Staphylococcus aureus* isolated from raw milk of cows with subclinical mastitis and *Staphylococcus aureus* isolated from the milking environment (blowers and milk conducting tubes). The results revealed that, in the presence of NaOCl (150ppm), the number of adhered cells of the twelve *S. aureus* strains was significantly reduced. When the same strains were evaluated in biofilm condition, different results were obtained. It was found that, after a contact period of five minutes with NaOCl (150ppm), four strains (two strains from milk , one from the blowers and one from a conductive rubber) were still able to grow. Although with the increasing contact time between the bacteria and the NaOCl (150ppm), no growth was detected for any of the strains. Concerning the efficiency of NaOCl on total biofilm biomass formation by each *S. aureus* strain, a decrease was observed when these strains were in contact with 150 ppm NaOCl for a total period of 10 minutes. This study highlights the importance of a correct sanitation protocol of all the milk processing units which can indeed significantly reduce the presence of microorganisms, leading to a decrease of cow´s mastitis and milk contamination.

INDEX TERMS: *Staphylococcus aureus,* mastitis infected cows, biofilm, sodium hypochlorite.

RESUMO.- [**Efeito do hipoclorito de sódio em biofilmes produzidos por *Staphylococcus aureus* isolados do ambiente de ordenha e de vacas com mastite.**] Biofilmes são constituídos de bactérias aderidas a uma superfície e aderidas entre si envolvidas por um polissacarídeo de constituição proteica, lipídica e glicídica que conferem uma barreira física às bactérias dentro deste microambiente. O objetivo deste trabalho foi analisar a eficácia do hipoclorito de sódio (NaOCl) contra estirpes de *Staphylococcus aureus* isoladas de leite cru de vacas com mastite subclínica e *Staphylococcus aureus* isolados do ambiente de ordenha (borrachas de ordenhadeiras e mangueiras condutoras de leite). Os resultados revelaram que, na presença de hipoclorito de sódio (150ppm), o número de células aderidas das 12 estirpes de *S. aureus* analisadas foi significativamente reduzido. Quando as mesmas estirpes foram avaliadas em condições de biofilme, diferentes resultados foram obtidos. Verificou-se que, após um período de contato de cinco minutos com NaOCl (150ppm), quatro estirpes (duas estirpes de leite, uma estirpe das borrachas das ordenhadeiras e uma estirpe de uma mangueira condutora de leite) ainda eram capazes de crescer. Com o aumento do tempo de contato do hipoclorito e as bactérias, cada vez maior, na concentração de 150ppm,

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não foi detectado o crescimento das estirpes. Em relação à eficácia do NaOCl na formação total da biomassa do biofilme por cada uma das estirpes de *S. aureus*, observou-se decréscimo da biomassa dos biofilmes quando estas estirpes estavam em contato com o NaOCl na concentração de 150ppm durante um tempo total de 10 minutos. O estudo demonstra a importância de um protocolo de saneamento correto de todas as unidades de processamento de leite, que pode, efetivamente, reduzir a presença de microrganismos de forma significativa, conduzindo a uma diminuição da mastite e da contaminação do leite.

TERMOS DE INDEXAÇÃO: *Staphylococcus aureus*, mastite bovina, biofilme, hipoclorito de sódio.

INTRODUCTION

Microbial communities attached to a surface and encapsulated in a self-produced polymeric matrix are known as biofilms. A particular characteristic of biofilms is their extreme tolerance to antimicrobial treatment. This tolerance is mediated by several mechanisms that can act together: (i) poor penetration or inactivation of antimicrobials inside the matrix, (ii) an altered bacterial metabolic state, (iii) the formation of persister cells, and (iv) resistance induced by the antimicrobial itself following the use of sublethal concentrations and the upregulation of efflux pumps (Mah & O'Toole 2001).

In fact, it is now of general consensus that sessile cells, simply adhered to a surface or already forming a biofilm, are much more tolerant to all common practice antimicrobial agents. In food industry, the efficacy of sanitizing protocols is of utmost importance. The most common used disinfectants to control microbial contamination during food processing are chlorine (or its derivatives), peroxygen, quaternary ammonium compounds (QACs) and acids, and their efficiency depends on the presence of waste, water hardness, temperature of application and the ability to contact with the microorganisms (Gibson et al. 1999).

The use of chlorine as a disinfecting agent is a common practice in dairy farms worldwide, since it is a good and cheap disinfecting agent. However, its low stability is a significant disadvantage as well as the non-compliance of the appropriate use criteria by the milk producers'. This fact can interfere with the quality of the disinfection process of the inner rubber liner, a very important factor in preventing mastitis. Mastitis is an inflammation of the mammary gland resulting from the colonization of pathogenic microorganisms, predominantly *Staphylococcus aureus*, *Streptococcus agalactiae*, coliforms, streptococci and enterococci (Amaral et al. 2004, Heringstad et al. 2000). This disease plays a major role in the microbiological quality of milk as it can modify the milk composition, increasing somatic cell counts and even causing cows' death, leading to large economic losses.

The objective of the present study was to evaluate the effectiveness of NaOCl at a concentration of 150ppm, throughout two different times of contact (5 and 10 minutes), against *S. aureus* strains isolated from milk of cows with subclinical mastitis and from sites in the milking environment, adhered or as biofilms, on polystyrene.

MATERIALS AND METHODS

Bacterial strains

Twelve strains of *S. aureus* isolated from cows with subclinical mastitis and milking environment (blowers and milk conducting rubber tubes) in a farm in Brazil were used in this research. The following tests were performed in the Laboratory of Applied Microbiology at the University of Minho, Braga, Portugal (Table 1).

Table 1. Source of the *Staphylococcus aureus* isolates used in the study

S. aureus strain	Source
106	Cow milk
123	Blowers
124	Hoses
160	Cow milk
162	Bulk tank milk
164	Blowers
166	Blowers
167	Blowers
174	Blowers
176	Blowers
179	Hoses
205	Cow milk

Study design and samplings

In this study we used samples of milk and milking environment (blowers and rubber tubes) collected on a milk farm in the state of Minas Gerais, Brazil. The cows were submitted to the *California Mastitis Test* (CMT) to identify the presence of subclinical mastitis, and then samples were collected from cows reagents to CMT. Swabs were also rubbed on the blowers and rubber tubes after the end of the afternoon milking, in order to isolate strains milking environment that could be correlated with the strains isolated from milk.

Swabs were placed in tubes containing sterile saline. Thus, all milk samples and swabs were placed in coolers with dry ice and sent for laboratory analysis at the Federal University of Uberlândia, Brazil. It was done the isolation and identification of strains of *Staphylococcus aureus* in milk of cows reagents to CMT as the swabs in the blowers and rubber conductive milk tubes. The strains identified as *Staphylococcus aureus* were sent to the laboratory of Applied Microbiology, University of Minho in Portugal for the tests about biofilm production.

The milking equipment was cleaned after each milking end with soap and water and disinfected with a solution containing 150ppm sodium hypochlorite. In this property was performed two milkings, one on morning and one on afternoon. Sampling was done in afternoon milking, before the process of cleaning and disinfection.

Effect of NaOCl on adhered cells of *Staphylococcus aureus*

Each *S. aureus* isolate was cultivated in Triptycase Soy Broth (TSB) for 24 hours at 37°C, with agitation (120rpm). Cells were harvested by centrifugation for 5 minutes at 10,500 g and 4°C followed by a washing procedure with a saline solution (0.9% NaCl in distilled water). After, the cells were homogenized in a vortex and the cellular suspension was adjusted with a saline solution to a final concentration of $1x10^9$ cells/mL determined by optical density at 640nm. To each well of a 96-well microplate, 200µL of the cellular suspension were added and incubated at 37°C for 2 hours, with agitation (120rpm). The wells were then carefully washed with saline solution (0.9% NaCl), and NaOCl was added at a concentration of 150 ppm and left in contact with the adhered cells

for 5 and 10 minutes under agitation at 37°C and 120rpm. The assay was performed in triplicate and in three different occasions.

Effect of NaOCl on *Stgaphylococcus aureus* biofilms

A *S. aureus* cellular suspension at a concentration of $1x10^6$ cells/mL was prepared in TSB supplement with 2% of glucose, according to the procedure described above. A volume of 200 µL of this suspension was pippeted into the wells of a 96-well microplate, which was then incubated with agitation (120 rpm) for 24h at 37°C. The biofilm formed on the surface of each well was washed twice with saline solution (0.9% NaCl) to remove planktonic cells. Finally, 200 µL of NaOCl at 150 ppm were added to each well and the microplates were incubated at 37°C and 120 rpm for 5, and 10 minutes. This assay was performed in triplicate, in three different occasions.

Colony forming units (CFU) enumeration

Adhered *S. aureus* cells and the biofilms formed in the microplate wells were washed with 0.9% NaCl followed by the addition of 200µL of the saline solution to each well, to help the scraping of the adhered cells and biofilms from the surface of each well. The resulting suspension was placed in a microtube and was sonicated for 20s at 30 W (Ultrasonic Processor, Cole-Parmer), with a diameter probe of 0.3cm, followed by a vigorous homogenization for 30s using a vortex. After homogeneization a serial dilution (10^{-1} to 10^{-6}) was performed. Aliquots of 10 µL of each dilution were drop plated, in triplicate, in Petri dishes containing TSA medium (Triptycase Soy Agar). The plates were incubated at 37°C for 48 hours. The resultant colonies were counted in each Petri dish.

Total biomass quantification (crystal violet assay)

To assess total biofilm biomass, after 24h of biofilm formation, and after treatment with NaOCl as described above (Gibson, et al. 1999), the microplate wells were washed with saline solution (0.9% NaCl) and fixed with 200 µL methanol in each well, which was removed after 15 minutes of contact. Then, the biofilm was allowed to dry at room temperature before adding 200 µL of crystal violet (1%v/v). After 5 minutes, the biofilm was gently washed with distilled water, and acetic acid (33% v/v) was added to each well (to release and dissolve the stain). The absorbance of the resulting solution was read in triplicate in an ELISA reader at 570nm.

Fluorescence microscopy

The bottoms of 6-well plates were cut into squares to be used as coupons for fluorescence microscopy assays. These coupons were placed in 6-well plates with 4mL of *S. aureus* cell suspension, at a concentration of $1x10^9$ cells/mL, and incubated at 37°C for 2 h with agitation (120rpm). Then the coupons were washed in saline solution (0.9% NaCl) and NaOCl was added at a concentration of 150 ppm and incubated at 37°C at 120rpm. The coupons were removed after 5 and 10 minutes of contact with the sanitizing agent. After removal, the coupons were gently washed with 0.9% NaCl and air-dried. The dried coupons were stained with 4´-6-diamidino-2-phenylindole (DAPI, Sigma, USA) solution 0.1 (g/L) during 30 minutes. Subsequently, each coupon was rinsed in distilled water in order to remove excess dye and let to air-dry in the dark for 30 minutes. Adhered cells were visualized under an epifluorescence microscope (Carl Zeiss, Germany) with a filter sensitive to DAPI fluorescence and coupled with a 3CCD video camera. For each coupon at least 10 images with an 820 x 560 resolution and 1000x magnification were taken.

Pulsed-Field Electrophoresis

The Pulsed-Field Electrophoresis assay was adapted from the McDougal et al. (2003) protocol. Briefly, a colony of *S. aureus* was grown in 5mL of Todd Hewit broth (THB) and incubated at 37°C for 24 hours under vigorous agitation. Afterwards, 1.5 mL of the bacterial suspension was centrifuged for 2 minutes at a 12 000rpm. The resulting pellet was resuspended in 500µL of TE (10mM Tris HCl, 1mM EDTA [pH 8.0]), heated at 37°C for 10 minutes, and centrifuged again at 12 000rpm for 2 minutes. The resultant pellet was resuspended once more in TE and the solution was heated at 45°C in the presence of 2% low melting agarose. Subsequently, a 20µL drop was placed in a glass slide covered with parafilm. The formed agarose discs were then carefully placed in 3mL of lysing solution (EC - 6mM Tris HCl, 1 M NaCl, 100mM EDTA, 0.5% Brij-58, 0.2% sodium deoxycholate, 0.5% lauroylsarcosine, pH 8.0) and 50µL of lysostaphin (1mg/mL), incubated at 37°C for 4 hours. After this period of time, the lysing buffer was removed and 4mL of fresh lysing solution with proteinase K (1mg/mL) and lysozyme (20µg/mL) were added. The new solution was incubated for 18 hours at 50°C.

After four washes with the lysing buffer at 37°C, for 30 minutes each, and incubation for 30 minutes at 25°C with a restriction buffer, the SmaI enzyme was added and left in solution for a period of 4 hours at 25°C.

After the digestion step, the DNA fragments were placed on the electrophoresis cell (CHEF-DR III - BioRad, Melville, NY) using a voltage of 200 V for 21 hours at 14°C.

The resulting gels were photographed and analyzed using Gel-Doc® (BioRad).

Pulsotype classification

The pulsotype classification was performed by the hierarchic group method, described by Sneath & Sokal (1973), using as similarity measurements the Jaccard coefficient and the Ward`s clustering algorithm (Ward 1963) to relate the groups. All the data was analyzed using R environment (R Development Core Team, 2010), 2.11.0 version. This assay was performed at the Departamento de Ciências Exatas da Faculdade de Ciências Agrárias e Veterinárias, Câmpus de Jaboticabal, SP, UNESP.

RESULTS AND DISCUSSION

The present study evaluated the effect of NaOCl (150ppm) on twelve strains of *Staphylococcus aureus* adhered to polystyrene (2h) and as biofilms (24h-old biofilm).

The number of adhered cells to the polystyrene surface varied according to the strain, ranging from $3.0x10^3$ to $2.4x10^6$ CFU/mL, as it can be seen in Table 2.

Table 2. Colony Forming Units (CFU) of 12 strains of *Staphylococcus aureus* adhered to polystyrene before and after treatment with NaOCl (150ppm)

S. aureus strain	CFU/mL before treatment	CFU/mL after treatment
106	$2.0x10^6$	ND
123	$2.4x10^6$	ND
124	$2.1x10^4$	ND
160	$2.1X10^6$	ND
162	$3.0x10^5$	ND
164	$8.0x10^3$	ND
166	$7.0x10^5$	ND
167	$5.0x10^5$	ND
174	$3.1x10^5$	ND
176	$1.2x10^5$	ND
179	$8.0x10^3$	ND
205	$3.0x10^3$	ND

ND = no colonies were detected.

The results revealed that NaOCl, was able to promote a significant reduction on the number of *S. aureus* cells adhered to polystyrene wells, regardless of the strain tested, the number of cells adhered, and the time of contact (bellow the detection limit).

The efficacy of 150ppm NaOCl against *S. aureus* adhered cells was also evaluated by fluorescence microscopy. As it can be seen in Figure 1, there is a significant decrease in the number of bacterial cells adhered to the polystyrene surface.

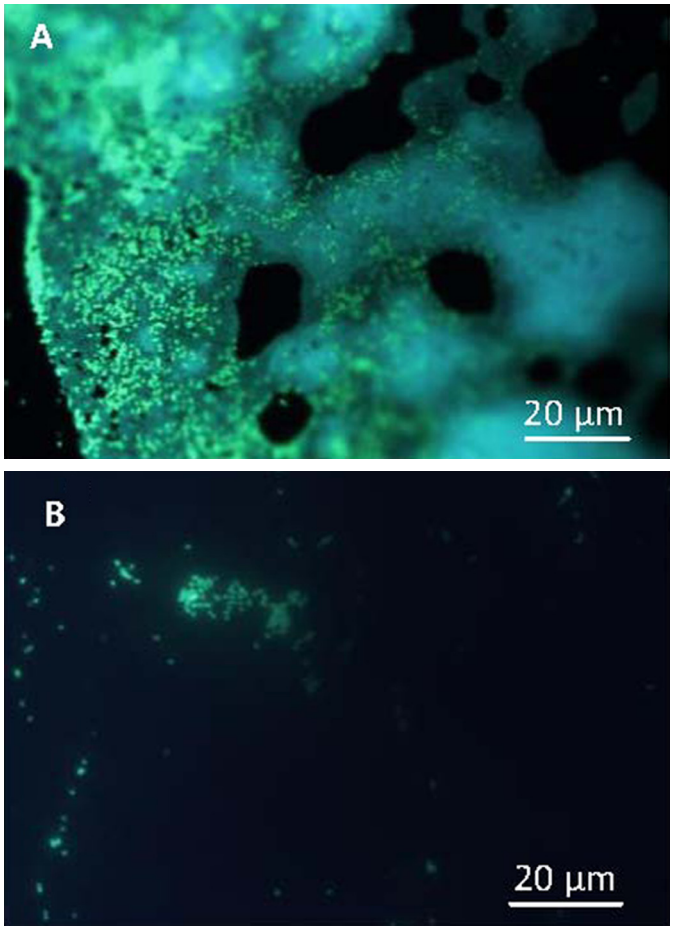

Fig.1. Fluorescence microscopy images showing the reduction of the number of *Staphylococcus aureus* cells adhered to polystyrene coupons after contact with 150ppm NaOCl for 5 minutes. *S. aureus* cells adhered to polystyrene coupons, (**B**) with and (**A**) without treatment with NaOCl.

When the sanitizing agent was used to treat already developed biofilms (24h-old), the degree of surface hygiene attained with 150ppm was dependent on the time of contact - only after 10 minutes no CFU were detected (Table 3). In fact, it was found that in four cases only a 2 log reduction in the number of CFU was obtained after 5 minutes of contact with NaOCl.

The different effect of sodium chloride on adhered cells compared to biofilms, after 5 minutes of contact, confirms that biofilm cells are highly tolerant to antimicrobial agents. In other words, biofilms constitute a shelter habitat that protects bacteria from detergents and sanitizers

Table 3. Colony Forming Units (CFU) obtained from 24h-old biofilms of 12 strains of *Staphylococcus aureus* before and after NaOCl (150ppm) treatment

S. aureus strain	CFU/mL before treatment	CFU/mL after treatement	
		5 min	10 min
106	$3.1x10^6$	ND	ND
123	$4.3x10^6$	ND	ND
124	$2.1x10^6$	ND	ND
160	$3.7x10^6$	$2.0x10^4$	ND
162	$4.2x10^6$	$2.5x10^4$	ND
164	$3.6x10^6$	ND	ND
166	$5.1x10^6$	ND	ND
167	$5.9x10^6$	ND	ND
174	$3.3x10^6$	ND	ND
176	$2.8x10^6$	$1.5x10^4$	ND
179	$3.2x10^6$	$2.1x10^4$	ND
205	$3.0x10^5$	ND	ND

ND = no bacterial growth was observed.

and where cells are able to develop specific traits like low multiplication rates or additional defense mechanisms, due to the ability of the extracellular polymeric matrix in neutralizing antimicrobial agents, since it consists of organic matter (Rossi & Porto 2009). This rationale is also in line with the results reported by Norwood & Gilmour (2000), after exposing multispecies biofilms (*Listeria monocytogenes*, *Pseudomonas fragi* and *Staphylococcus xylosus*) to increasing concentrations of NaOCl (200, 500 and 1000mg free chlorine/L for 20 minutes). The authors only obtained a 2 log CFU reduction in biofilm *L. monocytogenes* cells with the exposure to 1000mg chlorine/L, while 100% of the corresponding planktonic cultures were killed when exposed to 10mg chlorine/L for 30 seconds, confirming the shielding ability of biofilms. Also, according to the study of Amaral *et al*. (2004), where the efficiency of the disinfection of teatcups and teats during mechanic milking of dairy cows was tested, the disinfection of the teats with a 150ppm NaOCl soaking solution was able to significantly reduce the number of *Staphylococcus* sp. cells, while the immersion in the same solution was not efficient for the rubber teatcup microorganism reduction number. This demonstrates how biofilm formation on the surface of milking devices increases its resistance to antimicrobial agents.

Analyzing the strains whose biofilms were resistant to NaOCl at 150ppm, after 5 minutes treatment, and associating them to the pulsotypes identified by Pulsed-Field Electrophoresis (PFGE) (Table 4), it was found that three of the four resistant strains belong to the same pulsotype 63.

The strains used in the present study were isolated from cow's milk, the blower and from a hose. Curiously, the pulsotype 63 was isolated primarily from milk during the month of December 2011 and it was the only isolated from other two points, later, in January 2012. This is a strong indication that the hygiene protocol was failing, probably due to biofilm development, which is more resistant than adhered cells. Actually, after the blowers exchange, no strain with the pulsotype 63 was isolated. It is believed that the pulsotype 63 strain should have acquired resistance to NaOCl. A similar study by Bolton et al. (1988) showed that

Table 4. Pulsotype of the 12 strains of *Staphylococcus aureus*

S. aureus strain	Pulsotype	Source
106	52	Cow milk
123	4	Blowers
124	3	Hoses
160	63	Cow milk
162	66	Bulk tank milk
164	66	Blowers
166	67	Blowers
167	70	Blowers
174	67	Blowers
176	63	Blowers
179	63	Hoses
205	56	Cow milk

Table 5. Biofilm biomass of the twelve *Staphylococcus aureus* strains, isolated from cases of bovine subclinical mastitis and milking environment, expressed as crystal violet optical density ($O.D._{570\,nm}$), before and after exposure to NaOCl (150 ppm), for two different periods of time (5 minutes, 10 minutes)

S. aureus strain	$O.D._{570\,nm}$ before treatment	$O.D._{570\,nm}$ after treatment	
		5 min	10 min
106	0.35	0.51	0.07
123	0.79	0.48	0.05
124	0.70	0.61	0.05
160	0.79	0.77	0.06
162	0.42	0.42	0.04
164	0.55	0.66	0.23
166	0.59	0.67	0.06
167	0.60	0.69	0.08
174	0.59	0.70	0.71
176	0.64	0.63	0.76
179	0.67	0.65	1.30
205	0.87	0.92	1.10

strains of *S. aureus* endemic in equipment used in poultry processing, were eight times more resistant to chlorine than strains of *S. aureus* isolated from healthy skin.

In the current study, it was also observed an overall reduction of the total biofilm biomass, at a sanitizing concentration of 150ppm and contact time of 10 minutes (Table 5), which is accordance with the reduction on the number of viable cells at the same conditions.

A comparison of hypochlorite (alkaline hypochlorite), chlorine and chloramines has been performed for several times. It has been shown that chlorinated agents can penetrate into the biofilm of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Stewart et al. 2001)), but these chemicals are not able to inactivate all bacteria because the microorganisms in biofilms carry protective mechanisms against the lethal effect of this type of biocidal agents. In another study, it was observed that chloramines penetrated in the biofilm 6-8 times faster than hypochlorite. However, the bacteria that formed the biofilm were highly tolerant to both agents (Jang et al. 2006). These studies emphasize that the correct and conscious of cleanliness, hygiene, sanitation, and health education of workers must be daily and continuous, in addition to equipment maintenance and periodic exchange of rubbers. If these parameters are checked consistently, the installation process of the biofilm is hindered and, thus, the persistence of pathogenic bacteria with the constant contamination of the milk and the presence of mastitis in animals will be reduced and may even be eradicated.

CONCLUSION

This study shows that a correct sanitation protocol of all the milking installation can indeed significantly reduce the number of microorganisms' present, leading to a decrease of cow´s mastitis and milk contamination. Moreover, it is important to emphasize that the sanitation process must be adapted according to the strain of the microorganism detected.

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REFERENCES

Amaral L.A., Isa H., Dias L.T., Rossi-Jr O.D. & Nader-Filho A. 2004. Avaliação da eficiência da desinfecção de teteiras e dos tetos no processo de ordenha mecânica de vacas. Pesq. Vet. Bras. 24(4):173-177.

Bolton K.J., Dadd C.E.R., Mead G.C. & Waites W.M. 1988. Chlorine resistance of strains of *Staphylococcus aureus* isolated from poultry processing plants. Lett. Appl. Microbiol. 6:31-34.

Gibson H., Taylor J.H., Hall K.E. & Holah J.T. 1999. Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. J. Appl. Microbiol. 87:41-48.

Heringstad B., Klemetsdal G. & Ruane J. 2000. Selection for mastitis resistance in dairy cattle: a review with focus on the situation in the Nordic countries. Livest. Product. Sci. 64:95-106.

Jang A., Szabo J., Hosni A.A., Coughlin M. & Bishop P.L. 2006. Measurement of chlorine dioxide penetration in dairy process pipe biofilms during disinfection. Appl. Microbiol. Biotechnol. 72(2):368-376.

Mah T.F. & O'Toole G.A. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 9(1):34-39.

McDougal L.K., Steward C.D., Killgore G.E., Chaitram J.M., McAllister S.K. & Tenover FC. 2003. Pulsed-Field Gel electrophoresis typing of oxacilin-resistant *Staphylococcus aureus* isolates from the United: establishing a national database. J. Clin. Microbiol. 41(11):5113-5120.

Norwood D.E. & Gilmour A. 2000. The growth and resistance to NaOCl of *Listeria monocytogenes* in a steady-state multispecies biofilm. J. Appl. Microbiol. 88(3):512-520.

Rossi A.C.R. & Porto E. 2009. A importância da elaboração de procedimentos dehigienização considerando a presença de biofilmes. Sociedade Brasileira de Controle de Contaminação. Março/Abril 2009, p.40-41.

Sneath P.H. & Sokal R.R. 1973. Numerical Taxonomy: the principles and practice of numerical classification. W.H. Freeman, San Francisco. 573p.

Stewart P.S., Rayner J., Roe F. & Rees W.M. 2001. Biofilm penetration and disinfection efficacy of alkaline hypochlorite and chlorosulfamates. J. Appl. Microbiol. 91(3):525-532.

Ward J.H. 1963. Hierarchical grouping to optimize an objective function. J. Am. Statist. Assoc. 58:236-244.