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Full Length Research Paper

Comparative susceptibility of *in vitro* biofilm and planktonic cells of *Staphylococcus aureus* to antimicrobials

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This study evaluated the effect of frequently used veterinary wound antimicrobials for their efficacy in killing mature *in vitro Staphylococcus aureus* biofilms and inhibiting planktonic cells. The predictiveness of the minimum biofilm eradication concentration (MBEC) assay as a tool for antibiotic susceptibility testing was also assessed. The minimum inhibitory concentration (MIC) and MBEC of tetracycline, tetracycline-based commercial wound spray, silver nitrate, gentian violet, iodine tincture, sucrose and a laboratory mixture of sucrose and gentian violet were determined. Whereas low concentrations of all these antimicrobials except sucrose inhibited planktonic *S. aureus*, only silver nitrate eradicated the biofilm phenotype. Silver nitrate at a Ag^+ concentration of 4 x MIC showed 100% efficiency of removal or 7.70-log reduction of *S. aureus* biofilm cells, 1% gentian violet gave a significant reduction (55% or 0.35- log, P = 0.046) and 120% sucrose in gentian violet also showed a significant percentage reduction of 89.71% (0.98-log, P = 0.001). However, 120% sucrose and 2% iodine tincture reduced biofilms insignificantly (28.26% or 0.14-log, P = 0.098) and (34.78% or 0.18-log, P = 0.065), respectively. Based on the national committee for clinical laboratory brake-points, *S. aureus* biofilms lacked sensitivity to tetracycline and the tetracycline base wound spray. In conclusion, the antibiofilm properties of Ag^+ observed in this study may improve the success rate in treating clinical biofilm-associated *S. aureus* wound infections if the MBEC assay is applied to select appropriate concentrations.

Key words: *Staphylococcus aureus*, biofilms, planktonic, antimicrobials, wound, infections.

INTRODUCTION

Microbial biofilms are widely distributed in nature and are estimated to account for 65% of nosocomial infections costing the health care systems billions of dollars (Costerton et al., 1999; Mah and O'toole, 2001). Biofilms result when microbes come into contact with a surface in the presence of a fluid medium. Attachment is influenced by the type of surface, cells and relatively high nutrient fluxes in the medium as well as by other environmental factors (Kumar and Anand, 1998; Frank, 2000). Biofilm formation is a survival strategy by microbes in the natural environment to hostile conditions such as antibodies, phagocytes, desiccation and heat (Costerton et al., 1995). Studies have shown (Nickel et al., 1985; Mah and O'toole, 2001) that 10 - 1000 times more antibiotics are required to treat an infection caused by a biofilm-associated organism than a planktonic microbe of the same species. Treatment failure more often occurs as a consequence and this can have life threatening implications. Furthermore, biofilms also provide an ideal niche for the exchange of extra chromosomal DNA responsible for antibiotic resistance, making it a perfect milieu for emergence of drug resistant pathogens (Donlan, 2002).

Staphylococcus aureus account for 66% of all wound infections in animals (Tomlin et al., 2004) causing primarily difficult-to-treat biofilm-associated infections (James et al., 2007) which is a major challenge to healing of wounds. Acute infections may progress to become

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chronic with life-threatening implications depending on the site of the wound. Unfortunately, recent concern about the emergence of antibiotic resistant microbes and the limited new discovery of novel antibiotics within the last two decades limits treatment options for biofilm diseases. Antiseptics have been in use for much longer than antibiotics, yet resistance to antiseptics presents much less of a problem (Gilbert, 2006). They do not require prescription before use and are also cheaper as compared to antibiotics. However, the effect of common wound antiseptics on the biofilm phenotype has not been extensively investigated. Evidence indicate that mixtures of different types of antiseptics and mixtures of antiseptic with low concentrations of antibiotics are notable successful strategies for the control of biofilm associated wound infections (Carla and Carvalho, 2006).

Traditionally, the minimum inhibition concentration (MIC) which is based on the assumption that an antibiotic that is ineffective in preventing growth of a particular organism will also be clinically ineffective (Langston, 1999) has been used as the gold standard for determination of antimicrobial sensitivities (Costerton et al., 1995; Prescott and Baggott, 1985) . Evidence however indicates that a biofilm-associated organism that is sensitive *in vitro* to an antimicrobial may not be sensitive *in vivo*, meaning the MIC value for a particular antibiotic is not always predictive of clinical efficacy. Nevertheless, the MIC assay remains the only way that potentially effective antimicrobial agents are selected in most microbiology laboratories. Considering that biofilm formation is one of the major virulence factors involved in *S. aureus* wound infections, susceptibility testing of *S. aureus* should not rely on MIC determinations alone. The minimum biofilm eradication concentration (MBEC) assay could provide a quick and reliable methodology to assess the susceptibility of *S. aureus* cells growing in biofilms to antibiotics. The work described herein investigated the effects of frequently used topical antimicrobials (antibiotics and antiseptics) in modern veterinary wound care practice on *in vitro* biofilms and planktonic cells of *S. aureus*. This study also evaluated the reliability of the *in vitro* biofilm model and the MBEC assay for antimicrobial susceptibility testing for bacterial biofilms in the anticipation that the MBEC would be more reliable for selection of clinically effective antimicrobials.

MATERIALS AND METHODS

Inoculum preparation and formation of *in vitro* biofilm

S. aureus isolate obtained from an infected wound of a canine patient was used for this work. A 24 h culture of the *S. aureus* isolate was prepared on Baird Parker agar (Oxoid) and sub-cultured into 1% sterile peptone water to give an inoculum concentration of 10^8 cfu ml^{-1} (corresponding to 0.5 McFarland standards in turbidity). Biofilms were formed by aseptically inoculating 0.5 ml of inoculum onto glass slides as described by (Niemira and Solomon, 2005). Standard glass microscope slides (7.62 cm by 2.54 cm) were sterilized at 121°C, for 15 min and aseptically placed into 50 ml screw-capped centrifuge tubes containing 25 ml of 1% sterile peptone water. The slides were placed such that approximately 3.5 cm of the slide was submerged in culture medium and the upper part of the slide in the headspace of the tube remained dry. This was then inoculated with 0.5 ml of the freshly prepared inoculum. The experiment was carried out in duplicates and the tubes were held upright in a rack and incubated at 37°C for 48 h under static conditions. After 48 h of incubation, slide colonization was evaluated by washing off non adherent cells with physiological saline (10 ml per slide), by scraping of formed biofilm with sterile cotton wool swab into fresh sterile 1% peptone water, votexing and pour-plating. Slides containing approximately 10^6 to 10^7 bacteria growing as a biofilm following conditions developed from the procedure described above were used for antibiotic sensitivity test.

Preparation of antibiotic and antiseptic solutions

Antibiotics and antiseptics were identified and selected based on their common use in veterinary practice, human medicine and in research. Below are characteristics of the antimicrobials used.

Tetracycline, gentian violet, tincture of iodine and the commercial wound spray were obtained from a pharmacy shop whiles silver nitrate and sucrose was from Merck, Germany. Tetracycline, silver nitrate and sucrose were each weighed and dissolved aseptically in 50 ml centrifuge tubes containing 25 ml sterile 1% peptone water to obtain the different concentrations in (Table 1). Gentian violet and tincture of iodine were of 1 and 2% standard solutions respectively. The commercial wound spray was diluted from an original concentration of 20 mg/ml^{-1} tetracycline in gentian violet with sterile 1% peptone water to obtain concentrations tested in (Table 1) . A laboratory mixture of gentian violet and sucrose was prepared by weighing out desired weight of sucrose and dissolving in 50 ml centrifuge tubes containing 25 ml gentian violet to obtain concentrations desired.

Determination of minimum inhibitory concentration (MIC)

The MIC represents the lowest concentration of antimicrobial required to inhibit growth of a planktonic bacterial population. The MIC was determined from bacteria that were shed from the glass slides when they were placed in the differing concentrations of antimicrobials. Growth of shed bacteria in a particular tube was determined using the pour plate method. The lowest dilution at which shed bacteria failed to re-grow represented the MIC.

Minimum biofilm eradication concentration (MBEC) assay

Methods described by Merle et al. (2002) were modified and adopted for MBEC evaluation as below; 2-day old biofilms were used for antibiotic susceptibility testing. Merle et al. (2002) established that matured biofilms are produced at this level of colonization. Slides with attached bacterial biofilm were washed as above and transferred into 50 ml tubes containing diluted antimicrobial as above and incubated for 24 h at 37°C. The slide was then removed, rinsed in sterile physiological saline (0.85%) and placed in another tube containing fresh, sterile 1% peptone water. The remaining biofilm was removed from the slide first by scraping with a sterile cotton swab and then by vortexing at high speed for 3 min. This tube was incubated with the swab submerged in the peptone water for 24 h at 37°C after which the presence of viable bacteria was determined by the pour plate method. Growth of bacteria in a particular tube indicated re-growth of planktonic bacteria from surviving biofilm. The MBEC value represented the lowest dilution at which bacteria failed to re-grow. MIC and MBEC values were compared to values of the national committee for

Table 1. Characteristics of antimicrobials used in this study.

Name of antimicrobial	Group belonged to	Active ingredients	Concentrations tested
Commercial wound spray	Antibiotic and antiseptic mixtures	Tetracycline and Gentian violet	50, 40, 20, 10%
Tetracycline	Tetracyclines (antibiotic)	Tetracycline	15, 10, 4, 2, 1mg ml^{-1}
Gentian violet	Dyes (antiseptic)	Crystal violet	1%
Tinture of iodine	Iodines (antiseptic)	Potassium iodide, Iodine crystals.	2%
Silver nitrate	Metal ions (antiseptic)	Silver ions	1.27, 0.635, 0.32, 0.16%
Sucrose	(antiseptic)	Sucrose	120, 80 , 40%
Lab mixture	Antiseptic mixtures	Sucrose in Gentian violet	120, 80%

Table 2. MIC and MBEC of antimicrobials against 2 day old *S. aureus* biofilms.

Antimicrobial	MIC	MBEC	Antimicrobial brake-points
Tetracycline	< 1mg ml^{-1}	15mg ml^{-1}	R>0.16mg ml^{-1}<S
Wound spray	< 2mg ml^{-1}	10mg ml^{-1}	R>0.16mg ml^{-1} <S
Silver Nitrate	0.32%	1.27%	NA
Gentian Violet	1%	R	NA
Iodine	2%	R	NA
Sucrose	R	R	NA
Lab. Mixture	80%	R	NA

clinical laboratory standards (NCCL). The paired-samples T test of SPSS was used to analyze data and P = 0.05 was regarded as significant.

RESULTS

Biofilms of *S. aureus* readily formed on glass slides under the conditions described above. Estimates made of the size of the biofilm communities established at 48 h determined by plate count showed total viable attached populations of 5.0 x 10^7, 2.0 x 10^7, 4.6 x 10^7, 4.6 x 10^7 and 3.5 x 10^7 CFU/ml. Results of susceptibility test of these biofilm communities and planktonic cells of *S. aureus* growing in liquid culture media carried out in seven different antimicrobials with characteristics summarized in Table 1. The concentrations of antimicrobial required to inhibit planktonic *S. aureus* bacteria (MIC) and those required to kill the biofilm phenotype of the same organism (MBEC) are summarized in Table 2. The data represent the mean resulting from four replicate trials, r-means resistant that is, the concentration of antimicrobial used did not inhibit or kill all microbes and R-means resistance based upon the NCCLS brake points and S-means sensitive. NA- means no brake-points are available. After 24 h exposure of *S. aureus* to the seven antimicrobials tested, only silver nitrate at the concentrations mentioned above was able to kill and inhibit *S. aureus* biofilms and planktonic cells, respectively, (Table 2). Even then, the MBEC of silver nitrate of 1.27% was approximately 4 x MIC of 0.32% (Table 2) indicating that 4 times more silver was required to kill *S. aureus* biofilms than was required to inhibit *S. aureus* planktonic cells. 1% Gentian Violet and 2% iodine only inhibited planktonic cells but could not kill all biofilms of *S. aureus* (Table 2). Whereas the laboratory mixture inhibited planktonic cells, it failed to kill all biofilms, 120% sucrose in 1% peptone water could neither inhibit planktonic cells nor kill biofilms of *S. aureus* (Table 2). The results in Table 2 also show that tetracycline and the commercial wound spray both inhibited planktonic cells at < 1mgml^{-1} and < 2mgml^{-1} respectively, but based on NCCLS brake points for tetracycline, *S. aureus* biofilm was resistant to both antimicrobials. The efficiencies of antimicrobials in reducing the numbers of matured *in vitro* biofilm *S. aureus* cells on glass slides after 24 h exposure at 37°C are summarized in Table 3.

Data represent the means resulting from four replicate trials, GV- represents gentian violet. All concentrations in (Table 1) were tested for their efficiencies but only concentrations with the most percentage reduction were recorded in Table 3. Tetracycline and the commercial

Table 3. Efficiencies of antibiotics (in %) in reducing 2-day old *S. aureus* biofilms grown on glass slides.

		***Staphylococcus* population (cfu/ml)**			***Staphylococcus* reduction (log and %)**	
Antimicrobial	**Conc. (%)**	**Inoculums Conc. (A)**	**Before treatment (B)**	**After treatment (C)**	**$Log_{10}B$-$Log_{10}C$**	**{(B-C)/B} x100 (%)**
Silver	1.27	7.0×10^8 [a](8.84)	5.0×10^7 (7.70)	0.00 (0)	7.70	100
GV	1	4.0×10^8 (8.60)	2.0×10^7 (7.30)	9.0×10^6 (6.95)	0.35	55.00
Iodine	2	3.6×10^8 (8.55)	4.6×10^7 (7.66)	3.0×10^7 (7.47)	0.18	34.78
Sucrose	120	3.6×10^8 (8.55)	4.6×10^7 (7.66)	3.3×10^7 (7.51)	0.14	28.26
Lab mixture	120	1.8×10^8 (8.25)	3.5×10^7 (7.54)	3.6×10^6 (6.55)	0.98	89.71

[a] log cfu/ml.

wound spray were not included in efficiency analysis because *S. aureus* biofilms were found to be resistant to them (Table 2).

The data in Table 3 show that whereas only silver nitrate reduced biofilms of *S. aureus* by 100% or 7.70-logs, the laboratory mixture and 1% gentian violet both significantly reduced growth of *S. aureus* biofilms by 89.71% (0.98-log, P = 0.001) and 55% (0.35 -log, P = 0.046), respectively, (Table 3). Percentage reduction of growth shown by 2% iodine was 34.78% (0.18-log, P = 0.065) and 120% sucrose was 28.26% (0.14-log, P = 0.098) were however insignificant (Table 3) . The data presented in Table 3 provide evidence worth noting that sucrose combined with gentian violet at a concentration of 120% enhanced the percentage reduction of sucrose significantly from 28.26 (0.14-log) to 89.71% (0.98-log).

DISCUSSION

In the present study and in agreement with previous studies, (Merle et al., 2002; Krzyszlof et al., 2000; Hisonari et al., 1998) the biofilm phenotype of S. aureas was found to be more resistant to antimicrobials than planktonic cells. For example, whereas the MIC assay clearly indicated that all antimicrobials except sucrose were effective in inhibiting *in vitro S. aureus* cells, the MBEC data demonstrated that *S. aureus* biofilms were resistant to all antimicrobials except silver nitrate (Table 2). This observation agrees with clinical observations that *S. aureus* wound infections which are usually biofilm-associated require prolonged antimicrobial therapy and are frequently not responsive to treatment. The MBEC assay was therefore more predictive than the MIC assay. Silver nitrate at a low silver ion concentration of 4 x MIC demonstrated a 100% efficiency of removal or 7.70-log reduction of *S. aureus* biofilm cells (Table 3) in agreement with (Tomaselli, 2006; Babu et al., 2006) on the efficacy of silver ions as antimicrobial and with (Hisanori et al., 1998) on effect of silver ions on biofilms of *S. aureus*. However the concentration of silver in currently available wound dressings is much too low for treatment of chronic biofilm wounds (Bjarnsholt et al., 2007) . According to (Herbert et al., 2009) the efficiency of silver as a biocide in the ionic form even at low concentrations is due to its low propensity to select for resistance, its broad spectrum of activity and its high chemotherapeutic ratio.

This study found (Table 2) as in previous studies (Merle et al., 2002; Krzyszlof et al., 2000) tetracycline resistance in *S. aureus* biofilms based on the NCCLS brake-points of (MIC 0.16 $mgml^{-1}$) for tetracycline (NCCLS, 2000). This observation means that higher than accepted doses of tetracycline used in the pure form and mixed in the commercial wound spray have to be applied in clinical practice to treat *S. aureus* biofilms. This can result in treatment failure or overuse of antibiotics. Resistance to tetracycline in Staphylococcus spp has been identified to be mainly linked to acquisition of the plasmid- located genes tetK and tetL (Krzyszlof et al., 2000). The biofilm phenotype may have enhanced the spread of these genes by facilitating the horizontal transfer of plasmids.

The activity of sucrose as an antiseptic is based on the environment of low water activity it creates when dissolv-

ed in water which inhibits bacterial growth (Chirife and Herszage, 1982). The observation that 120% sucrose in 1% peptone water did not inhibit *S. aureus* planktonic cells (Table 2) or reduce bioflm cells of *S. aureus* significantly (28.26% or 0.14-log, P = 0.098) (Table 3) indicated that probably the concentration of sucrose used was not high enough to create the required water activity. This was shown by Hisonari et al. (1998) who found that 70% sucrose in plasma could not significantly inhibit or kill *S. aureus* biofilms and by Chirife and Herszage (1982) who observed growth of *S. aureus* in sugar concentration of <183 g of sugar / 100 g of water but a complete inhibition of growth at a concentration of 195 g of sucrose / 100 g of water. Sucrose when mixed with gentian violet in the laboratory at a concentration of 120% sucrose enhanced the percentage reduction of sucrose significantly (89.71% or 0.98 -log, P = 0.001) (Table 3) suggesting the additional inhibitory property of gentian violet may have accounted for the enhanced antiseptic activity of sucrose and in agreement with (Carla and Carvalho, 2006). According to Carla and Carvalho (2006) mixtures of biocidal agents usually contain effective amounts of a detergent to decrease the surface tension of the biofilm, a denaturing agent to attack the extra cellular matrix and a biocide to kill the micro-organisms as such they are one of the most efficient solution to destroy biofilms.

A significant percentage reduction of 55% (0.35-log reduction, P = 0.046) of *in vitro S. aureus* biofilms was achieved by 1% gentian violet (Table 3) parallel to observations by Sousa et al. (2008) who noted that the use of gentian violet as an antiseptic prevented bacterial proliferation on surfaces but did not eradicate biofilms. According to (Saji et al., 1995) gentian violet shows good bactericidal activity against gram-positive cocci (e.g., *Staphylococcus* species) and pathogenic yeasts such as *Candida* spp and this may have accounted for the significant reduction of *S. aureus* biofilms observed in this study. This activity of gentian violet was further enhanced when it was mixed with sucrose (Table 3).

The present study observed that *S. aureus* biofilms were resistant to 2% iodine tincture (Table 2) and that the percentage reduction of 34.78% (0.18-log reduction, P = 0.213) (Table 3) was also not significant. Generally, the antimicrobial efficacy of iodine is said to be debatable (Kramer, 1999) and its toxicity to host tissue is also a matter of concern. Whereas (Presterl, 2007) observed that tincture of iodine was not as effective as some other biocides in eradicating *in vitro* biofilms of *Staphylococcus epidemidis*.

Elisabeth et al. (2007) obtained a 5-log bacterial reduction within the biofilms of *S. epidemidis* with tincture of iodine but a low number of viable bacteria persisted. Results obtained in this study indicate that the use of 1% gentian violet, 2% tincture of iodine, sucrose and a mixture of sucrose with gentian violet at a concentration of 120% sucrose in the treatment of *S. aureus* wound infec-tion will result in a persistent infection that may become chronic.

Generally much of biofilm-associated antibiotic resistance observed in this study and in previous work (Costerton et al., 1999; Mah and O'toole, 2001; Nickel, 1985) can be attributed to:

(1) The extra cellular polymeric substances (EPS) secreted by biofilm bacteria, acts as a physical/chemical barrier, thus preventing penetration by many antibiotics (Thien and O'toole, 2001).
(2) EPS is negatively charged and functions as an ion-exchange resin which is capable of binding a large number of the antibiotic molecules that are attempting to reach the embedded biofilm cells (Prakash et al., 2003).
(3) Embedded biofilm bacteria are generally not actively engaged in cell division, are smaller in size and less permeable to antibiotics (Thien and O'toole, 2001). Virtually all antimicrobials are more effective in killing rapidly-growing cells.

Conclusion

In conclusion, this study has shown that: Gentian violet enhanced significantly the antimicrobial activity of sucrose and vice versa against *S. aureus* biofilms Silver nitrate at a Ag^+ concentration of 4 x MIC exhibited the most efficient removal of *in vitro S. aureus* biofilm cells. The MBEC assay is indeed more predictive than the MIC assay in antibiotic susceptibility testing. With a rising concern of the spread of antibiotic resistant microbes, the bactericidal and antibiofilm properties of Ag^+ observed in this study may improve the success rate in treating clinical biofilm-associated *S. aureus* wound infections if the MBEC assay is applied to select appropriate concentrations.

REFERENCES

Babu R, Zhang J, Beckman EJ, Virji M, Pasculle WA, Wells A (2006). Antimicr,obial activities of silver used as a polymerization catalyst for a wound-healing matrix, Biomater., 27: 4304-4314.

Bjarnsholt T, Kirketerp-Møller K, Kristiansen S (2007).Silver against Pseudomonas aeruginosa biofi lms. AMIS. 115: 921-928.

Carla CC, De Carvalho R (2006) Biofilms: Recent Developments on an Old Battle. IBB–Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

Costerton JW, Stewart PS, Greenberg EP (1999). Bacterial biofilms: a common cause of persistent infect. Sci. 284: 318-322.

Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM (1995). Microbial biofilms. Ann. Rev. Microbiol. 49: 711–745.

Chirife J, Herszage L (1982). Sugar for infected wounds. Lancet ii, pp. 157.

Donlan RM (2002). Emerg. Infect. Dis; 8: 881-890.

Elisabeth P, Miranda S, Michaela E, Sonja R, Andrea L,Wolfgang G, Manfred R (2007). Effects of alcohols, povidone-iodine and hydrogen peroxide on biofilms of Staphylococcus epidermidis. J. Antimicrob. Chemother. 60: 417-420.

Frank JF (2000). Microbial attachment to food and food contact surfaces. Adv. Food, Nutr. Res. 43: 319-370.

Gilbert P (2006). Avoiding the resistance pitfall in infection control.

Ostomy Wound Manage., 52(10): 1-3.
Herbert NP, Daniel LP (2009) Antimicrobial Silver in Orthopedic and Wound Care Products. Rodman Online Publications.
Hisonari A, OsamuY, HirokoK, Joji T, Jiro A (1998).Effects of sucrose and silver on *Staphylococcus aureus* biofilms. J. Antimicrob. Chemother., 42: 629-634.
James GA, SwoggerE, Wolcott R, DeLancey Pulcini E, Secor P, Sestrich J, Costerton JW, Stewart PS (2007) . Biofilms in Chronic Wounds. Wound Repair and Regeneration. In Press.
Kramer SA (1999). Effect of iodine on wound healing: a review. J. Vasc. Nurs. 17: 17-23.
Krzyszlof T, Ben SC, Waleria H, Christopher GD (2000). Expression of resistance to tetracyclines in strains of mithicillin-resistant *Staphylococcus aureus*. J. Antimicrob. Chemother., 45: 763-770.
Kumar CG, Anand SK (1998). Significance of microbial biofilms in food industry: a review. Int. J. Food Microbiol., 42:9-27.
Langston VC (1999). Antimicrobial use in food animals. In: Howard JL, Smith RA, eds. Current Veterinary Therapy 4: Food Animal Practice. Philadelphia: WB Saunders, pp. 17-32.
Mah TFC, OO'toole GA (2001) Mechanisms of biofilm resistance to antimicrobial agents. Trends. Microbiol., 9: 34-39.
Merle EO, HC Douglas W, Morck K, Andre G, Ronald RR (2002). Biofilm bacteria: formation and comparative susceptibility to antibiotics. Can. J. Veter. Res., 66: 86-92.
NCCLS - National Committee for Clinical Laboratory Standards (2000), Approved standard M7-A5. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 5. ed. NCCLS, Wayne, Pa.
Nickel JC, Ruseska I, Wright JB, Costerton JW (1985). Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. Antimicrob Agents Chemother., 27: 619-624.
Niemira BA, Solomon EB (2005). Sensitivity of Planktonic and Biofilm-Associated *Salmonella* spp. to Ionizing Radiation. Appl. Environ. Microbiol. 71: 2732-2736.
Prakash B, Veeregowda BM, Krishnappa G, (2003). Biofilms: A survival strategy of bacteria**.** Current Sci. 85: 9, 10.
Prescott JF, Baggott JD (1985). Antimicrobial susceptibility testing and antimicrobial drug dosage. J. Am. Vet. Med. Assoc. 187: 363-368.
Presterl E, Suchomel M, Eder M (2007). Effects of alcohols, povidone-iodine and hydrogen peroxide on biofi lms of Staphylococcus epidermidis. J. Antimicrob. Chemother., 60: 417- 420.
Saji M, Taguchi S, Uchiyama K, Osono E, Hayama N, Ohkuni H. (1995). Efficacy of gentian violet in the eradication of methicillin-resistant *Staphylococcus aureus* from skin lesions. J. Hosp. Infect., 31: 225-228.
Sousa C, Henriques M, Teixeira P, Oliveira R (2008). Reduction of Staphylococcus epidermidis adhesion to indwelling medical devices: a simple procedure. Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar, Braga, Portugal. pp. 4710-057.
Thien FCM, O'toole GA (2001). Trends Microbiol., 9: 34-39.
Tomlin J (2004). Methicillin-resistant *Staphylococcus aureus* infections in 11 dogs. Veterinary Record. 144: 60-64
Tomaselli N (2006). The role of topical silver preparations in wound healing. J. Wound. Ostomy. Continence Nurs., 33: 367-380.