



TURNING NOVEL DISCOVERIES INTO
INNOVATIVE HEALTHCARE SOLUTIONS

Aledex™: A Novel Antimicrobial-Antibiofilm Technology

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1. Aledex™ a PS/CHX Combination

Kane Biotech has developed a broad-spectrum anti-infective composition PS/CHX comprising two FDA-approved compounds protamine sulfate (PS) and chlorhexidine (CHX) (U.S. Patent Publication No.20070003538 A1 and PCT International Publication No. WO/2007003028 A1). Protamine sulfate and CHX are combined because the former facilitates the transport of antimicrobial compounds to the cytoplasm (Antohti and Popescu, 1979) and the latter is a broad-spectrum antimicrobial agent that has been demonstrated to successfully prevent clinical infections associated with catheters (Maki *et al.*, 1997).

Protamine sulfate alters the permeability of microbial cell membrane and the dilation of ion channels, which facilitate the transport of antimicrobial compounds to the cytoplasm. In addition, antimicrobial activity of PS is presumably due to electrostatic attraction between the positively charged molecule and the negatively charged cell envelope, and appropriate concentrations cause growth inhibition or cell death because of leakage of K⁺, ATP and intracellular enzymes (Johansen, *et al.*, 1997). Furthermore, PS has been reported to prevent *Staphylococcus* sp. adhesion to stainless steel (Matsumura, *et al.*, 2007). Presumably, PS impairs bacterial biofilms by binding to polysaccharides and disrupting the microbes' hydrophilic surface coat (Teichman, *et al.*, 1994). Chlorhexidine is a membrane-active agent, causing protoplast and spheroplast lysis. At a higher concentration, it causes precipitation of proteins and nucleic acids (McDonnell and Russell, 1999). When PS is used in combination with CHX, it facilitates the transport of CHX to cytoplasm and thus reduces the effective concentration of CHX (Darouiche, *et al.* 2008). Protamine sulfate has been shown to enhance both *in vitro* and *in vivo* antimicrobial as well as antibiofilm activities of antibiotics and non-antibiotic compounds (Burton, *et al.*, 2006; Hansen, *et al.*, 2001; Richards, *et al.*, 1990; Soboh, *et al.*, 1995; Teichman, *et al.*, 1994; Yakandawala, *et al.*, 2007; U.S. Patent No. 7,144,992; U.S. Patent No. 7,314,857; Canadian Patent No. 2452032).

2. History of Use

2.1 Protamine Sulfate (PS)

The use of PS in the neutralization of heparin is well known and well established. The drug has been successfully used for almost 50 years. Heparin is used to prevent blood clots from forming. Protamine sulfate is given when there is an excessive bleeding from heparin administration

(Jaques, 1973; Lindblad, 1989). Protamine reverses the effect of heparin by dissociation of heparin-antithrombin III complexes. Protamine-heparin complexes are formed, and thereby the amplified inhibition of the coagulation by heparin is normalized. It has been approved by the FDA for injection or infusion into the blood vessel to treat an overdose of heparin. It is currently marketed by Eli Lilly.

2.2 Chlorhexidine (CHX)

Chlorhexidine containing compounds have been used as topical disinfectants since the middle 1970's. As an effective broad-spectrum antimicrobial agent, CHX subsequently found use in oral care products such as mouth rinses and toothpaste (Peridex, Periochip, PerioGard, Chlorohex, Savacol, etc.), and antiseptic skin creams, and disinfectants used to prepare the skin for surgical procedures (Oronine, Avagard, Hibiclens, Hibiscrub, ChloroPrep, and Exidine). It is also a component of the famous household antiseptic Savlon. In the early 1990's, the FDA cleared the following three medical devices containing CHX: (i) intravenous catheters (ARROWg+ard Blue and ARROWg+ard Blue Plus), (ii) topical antimicrobial skin dressings (BIOPATCH and BACTIGRAS), and (iii) an implanted surgical mesh (GORE DUALMESH® PLUS) (FDA Public Health Notice <http://www.fda.gov/cdrh/chlorhex.html>).

3. Efficacy

3.1 In vitro efficacy

3.1.1 Minimal inhibitory concentration (MIC)

The MICs of PS and CHX alone and PS/CHX combination for catheter-associated bacteria and yeast were determined (Amsterdam, 1996). The MICs of CHX ranged from 0.195 to 12.5 µg/ml as compared to 3.25 to >200 µg/ml for PS (**Table 1**). The PS/CHX combination showed synergistic inhibitory effects on uropathogens *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and Vancomycin-resistant *Enterococcus faecium* (VRE). The MIC values of CHX for these organisms in the presence of PS were reduced by about 50%.

Table 1: MIC (µg/ml) of PS and CHX alone and the PS/CHX combination for catheter associated pathogens ^a

Organism	MIC (µg/ml)		
	PS	CHX	PS/CHX
<i>Escherichia coli</i>	> 100	0.39	0.39/0.195
<i>Klebsiella pneumoniae</i>	> 200	6.25	12.5/3.125
<i>Proteus mirabilis</i>	> 200	12.5	50/12.5
<i>Pseudomonas aeruginosa</i>	200	12.5	50/12.5
<i>Staphylococcus epidermidis</i>	3.125	0.195	0.39+0.195
<i>Staphylococcus aureus</i>	200	0.781	1.56/0.39
<i>Enterococcus faecalis</i>	> 100	3.125	6.25/3.125
<i>Vancomycin-resistant Enterococcus faecium</i>	12.5	0.781	1.56/0.39
<i>Candida albicans</i>	> 100	3.125	6.25/3.125

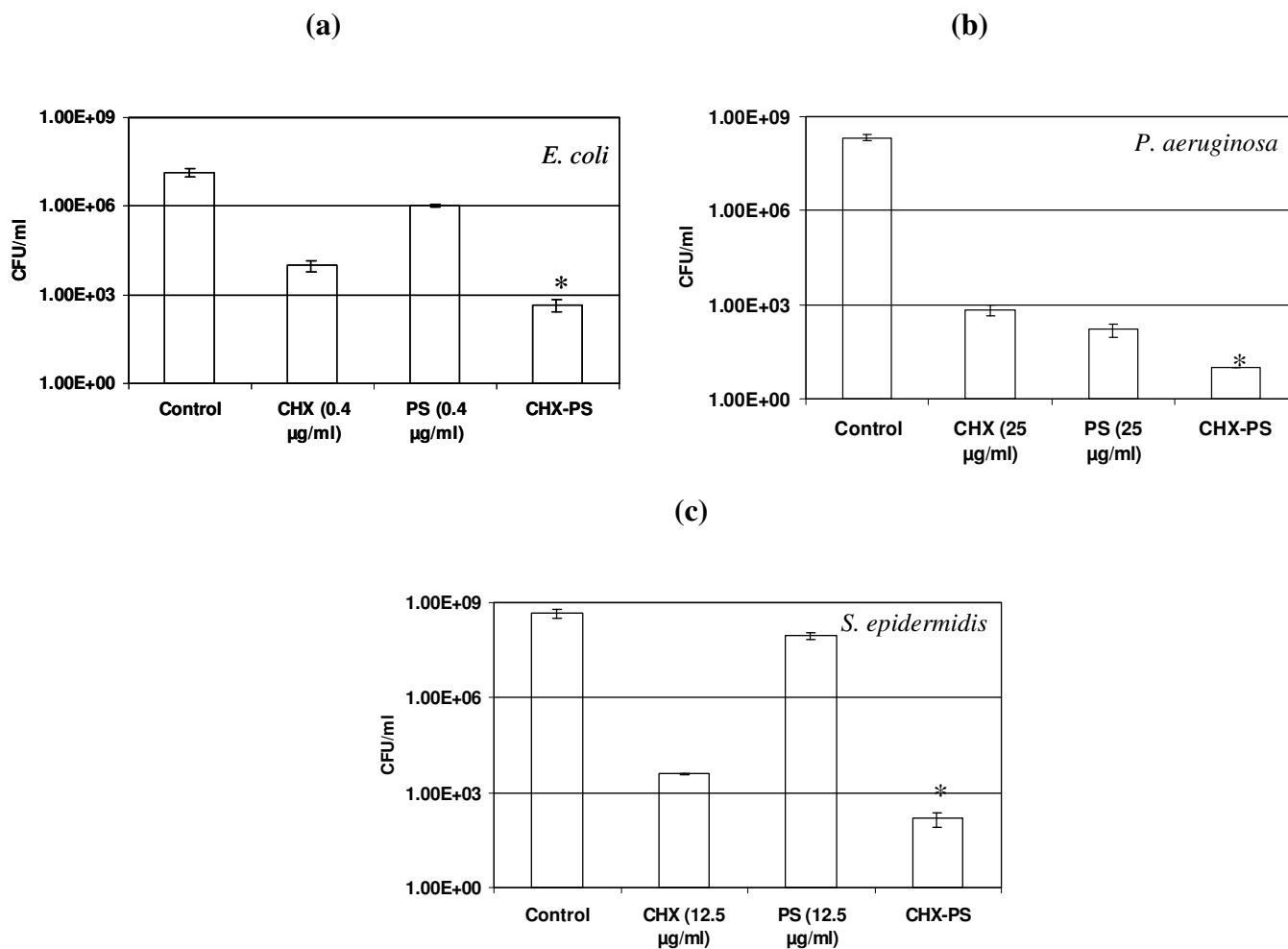
^a As determined by microtiter broth dilution method. The initial inoculum was adjusted to 10⁵ CFU/ml in tryptic soy broth.

3.1.2 Antibiofilm activity

Biofilm was developed in 12-well assay plate in the presence of PS and CHX alone and in combination for 24 h. After 24 h incubation at 37°C, medium containing planktonic cells was discarded and the biofilm was washed once with the physiological saline. After disrupting the washed biofilm by sonicating the plate for 1 min, biofilm-embedded cells were serially diluted in saline and plated on Tryptic Soy Agar (TSA) plates to estimate the total viable count (TVC). The antibiofilm activity of PS and CHX alone and PS/CHX combination for catheter-associated bacteria and *C. albicans* was determined. PS in combination with CHX showed a significant synergistic inhibitory effect on biofilm formation in *E. coli*, *P. aeruginosa* and *S. epidermidis* ($P < 0.05$) (**Fig. 1**). *P. aeruginosa* biofilm formation was completely inhibited when PS and CHX were used together at a concentration of 25µg/ml each. Although the synergy between PS and

CHX was not observed against biofilm formation in *K. pneumoniae*, *E. faecalis* and *C. albicans*, PS/CHX combination inhibited more than 90% biofilm formation (data not shown).

Fig. 1: Effect of normal saline control, PS and CHX alone and PS/CHX combination on biofilm formation in (a) *E. coli*, (b) *P. aeruginosa* and (c) *S. epidermidis*.



3.1.3 Antimicrobial activity of PS and CHX coated urinary catheters

The overnight grown cultures were spread on to the surface of Mueller-Hinton agar plates and 1 cm segments of silicone catheter coated with PS/CHX combination were placed on to the surface of the agar plate (Sherertz, *et al.*, 1989). The plates were incubated at 37°C for 24 h and zone of inhibition was measured as a clear zone perpendicular to the long axis of the catheter segment. Our *in vitro* zone of inhibition assay data (**Table 2**) indicates that catheters coated with PS/CHX combination were active at inhibiting the growth of catheter-associated pathogens.

Table 2: *In vitro* antimicrobial activity of PS/CHX-coated urinary catheters^a

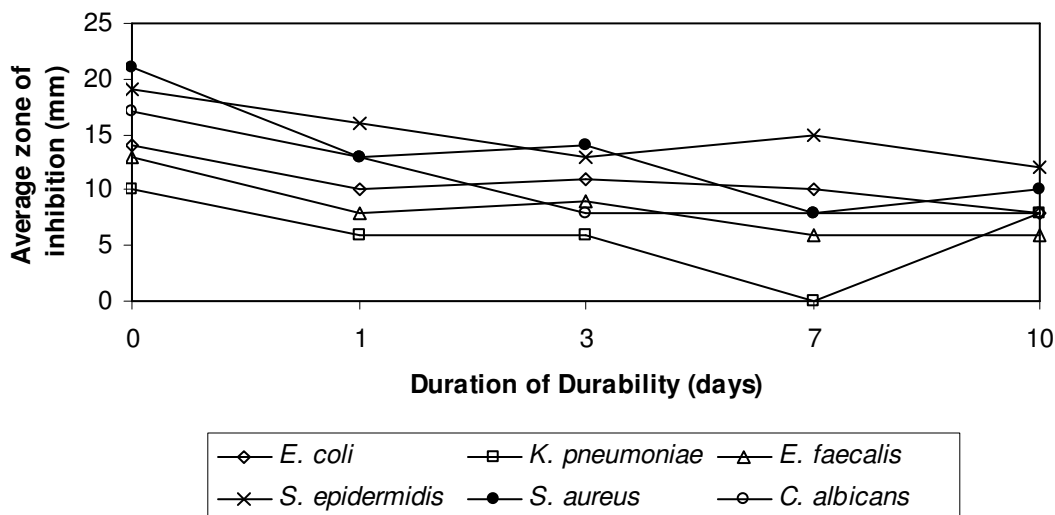
Pathogen	Mean zone of inhibition (mm)
<i>E. coli</i>	14
<i>K. pneumoniae</i>	13
<i>P. mirabilis</i>	8
<i>Acinetobacter calcoaceticus</i>	7
<i>P. aeruginosa</i>	6
<i>S. epidermidis</i>	14
<i>S. aureus</i>	21
<i>E. faecalis</i>	13
Vancomycin-resistant <i>E. faecium</i> (VRE)	13
<i>C. albicans</i>	17

^aA suspension of bacteria (10^8 CFU/ml) was streaked with cotton swab on Mueller-Hinton agar plates. Plates were incubated at 37°C and zones of inhibition were measured the following day or after 48 hours for *C. albicans*

3.1.4 *In vitro* durability of PS/CHX coated urinary catheter as determined by zone of inhibition

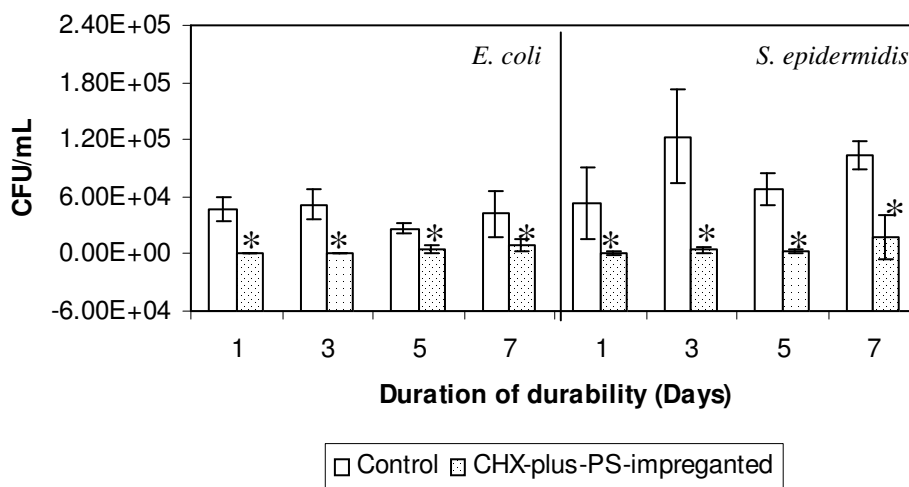
The overnight grown cultures were spread on to the surface of Mueller-Hinton agar plates and 1 cm segment of silicone catheter coated with PS/CHX was placed on to the surface of agar plate. The plates were incubated at 37°C for 24 h and zone of inhibition was measured as a clear zone perpendicular to the long axis of the catheter segment. The catheter segments were transferred daily to fresh lawns on Mueller-Hinton agar. The PS/CHX-coating was effective in inhibiting the growth of test organisms for more than 10 days (**Fig. 2**). The urinary catheters coated with the combination of PS and CHX provide *in vitro* durability against a variety of Gram-negative bacilli (*E. coli* and *K. pneumoniae*) and Gram-positive cocci (*E. faecalis*, *S. epidermidis*, and *S. aureus*) as well as *C. albicans*.

Fig. 2: *In vitro* durability of PS/CHX-coated urinary catheters as determined by zone of inhibition



3.1.5 *In vitro* durability of PS/CHX-coated catheter in artificial urine medium

The uncoated and PS/CHX-coated urinary catheters were incubated in artificial urine medium separately for 7 days at 37°C with 100 rpm shaking. On day 1, 3, 5, and 7 the ability of PS/CHX-coated catheter segments to resist colonization by *E. coli* and *S. epidermidis* was studied using adhesion assay (Cormio, *et al.*, 2001). Catheter segments were removed on day 1, 3, 5, and 7 and rinsed in 1 ml phosphate buffer saline (PBS) and placed in 10 ml BHI broth. The tubes were inoculated with 100 μ l of bacterial suspension (10^7 CFU) and incubated in a water bath for 3 h at 37°C with gentle shaking. After incubation, the catheter segments were washed, sonicated, vortexed and serial dilutions of bacterial cells was plated onto LB agar plates. Plates were incubated at 37°C for 24 h, and colonies were counted. The antimicrobial coating of catheters prevented $\geq 80\%$ of *E. coli* and *S. epidermidis* colonization after soaking the catheters in sterile artificial urine medium (**Fig. 3**). The coated catheters exhibited significantly less colonization ($P < 0.05$) by *E. coli* and *S. epidermidis* compared to uncoated control. Retention of antimicrobial activity for 7 days showed the antimicrobial agents were not burst released into the medium at the very beginning.

Fig. 3: *In vitro* durability of PS/CHX-coated urinary catheters in synthetic urine

3.2 *In vivo* efficacy

3.2.1 *In vivo* efficacy of PS/CHX coated urinary catheters in a rabbit model of *E. coli* infection

The catheter segments uncoated, PS/CHX-coated, and silver-hydrogel-coated were subcutaneously inserted in 14 rabbits and the insertion sites were inoculated with clinical isolates of *E. coli* (Darouiche, *et al.*, 2002). After one week rabbits were sacrificed and catheter segments and swab cultures of the site adjacent to catheter segment were obtained for bacteriological analysis. All 14 rabbits tolerated surgery well and exhibited no evidence of sepsis or failure to thrive, indicating the safety of the antimicrobial compounds. As Table 3 shows, 2/28 (7%) PS-plus-CHX coated catheters, 25/28 (89%) silver-hydrogel coated catheters, and 18/28 (64%) uncoated catheters became colonized with *E. coli*. The PS/CHX-impregnated catheters were significantly less likely to be colonized than either silver hydrogel-coated catheters ($P < 0.001$) or uncoated catheters ($P < 0.001$), and there was no significant difference ($P = 0.055$) in the rate of colonization of silver hydrogel-coated vs. uncoated catheters. Furthermore, 1/28 (4%) PS/CHX-impregnated catheters, 12/28 (43%) silver hydrogel-coated catheters, and 14/28 (50%) uncoated catheters resulted in infection due to *E. coli*. The PS/CHX-impregnated catheters were significantly less likely to cause infection than either silver hydrogel-coated catheters ($P = 0.001$) or uncoated catheters ($P < 0.001$), and there was no significant difference ($P = 0.79$) in the incidence of infection caused by silver-hydrogel-coated vs. uncoated catheters.

Table 3: *In vivo* efficacy of PS/CHX-coated and uncoated urinary catheters against *E. coli*^a

Outcome	No. of catheter segment with outcome/total (%)			P value
	PS/CHX	Silver hydrogel-coated	Uncoated	
Catheter colonization	2/28 (7%)	25/28 (89%)	18/28 (64%)	< 0.001 ¹ < 0.001 ² 0.055 ³
Catheter-related infection	1/28 (4%)	12/28 (43%)	14/28 (50%)	0.001 ¹ <0.001 ² 0.79 ³

^aCatheter segments (2 cm long) were subcutaneously inserted in rabbits and *E. coli* suspension (10⁵ CFU) was inoculated at the insertion site. After seven days, catheter segments and surrounding swab cultures were analyzed for catheter colonization and related infection.

¹ P value for PS/CHX-impregnated vs. silver-hydrogel-coated catheter

² P value for PS/CHX-impregnated vs. uncoated catheter

³ P value for silver- hydrogel-coated vs. uncoated catheter

4. *In vitro* antimicrobial activity of PS/CHX against bacteria associated with microbial contamination in industries

E. coli, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, *Streptococcus thermophilus*, *Listeria monocytogenes* and *Clostridium perfringens* are bacteria frequently encountered in a wide variety of industries, including dairy, pulp and paper mills, food and beverage manufacturing industry, water treatment facilities, etc. Some of them are commonly found in a variety of consumer products and household items, and are often found in, for example, kitchens, bathrooms, HVAC systems, humidifiers, vacuum cleaners, toys and the like.

4.1 Minimal inhibitory concentration (MIC)

The MICs of PS and CHX combination for *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, *Streptococcus thermophilus*, *Listeria monocytogenes* and *Clostridium perfringens* was performed as described in Section 3.1.1. As shown in Table 1 and 4, the combination of PS/CHX was active against all the pathogens tested.

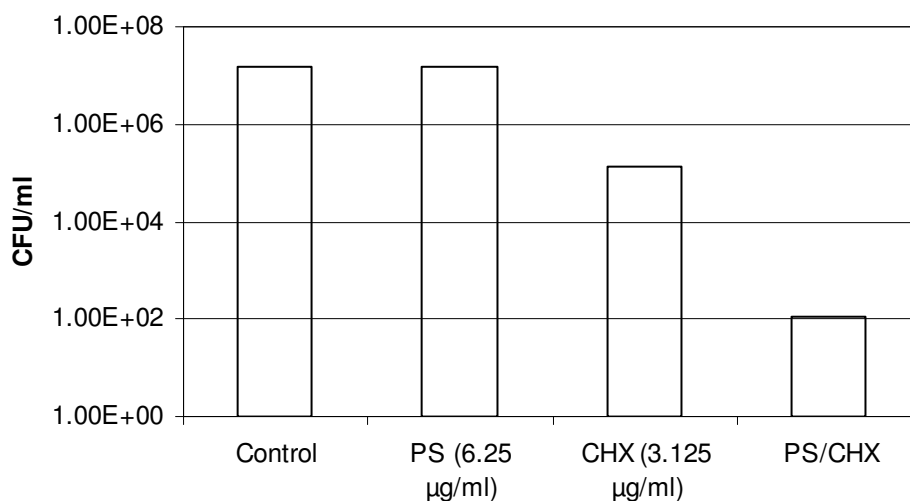
Table 4 MIC ($\mu\text{g/ml}$) of PS and CHX combination for bacteria associated with food and dairy industries^a

Organism	MIC ($\mu\text{g/ml}$) of PS/CHX combination
<i>Bacillus cereus</i>	12.5/3.125
<i>Streptococcus thermophilus</i>	< 0.195/0.78
<i>Listeria monocytogenes</i>	12.5/3.125
<i>Clostridium perfringens</i>	3.25/1.56

4.2 Antibiofilm activity of PS/CHX

The antibiofilm activity of PS/CHX was tested against *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, *Streptococcus thermophilus*, *Listeria monocytogenes* and *Clostridium perfringens* as described in Section 3.1.2. The combination of CHX and PS was synergistic in inhibiting the growth of biofilm embedded *P. aeruginosa*, *E. coli* and *Bacillus cereus* (Figs 1 and 4). Although the synergy between PS and CHX was not observed against biofilm formation in *K. pneumoniae*, *S. aureus*, *S. thermophilus*, *L. monocytogenes*, and *C. perfringens* PS/CHX combination inhibited more than 90% biofilm formation (data not shown).

Fig. 4: Effect of normal saline control, PS and CHX alone and PS/CHX combination on biofilm formation in *Bacillus cereus*.



5. *In vitro* antimicrobial activity of PS/CHX against oral pathogens associated with plaque, caries and periodontal diseases in humans and animals

Streptococcus mutans and *Streptococcus sobrinus* are the major oral bacteria associated with dental caries. They are the primary colonizers of teeth resulting in early dental plaque formation. The other oral bacteria such as *Actinobacillus naeslundii*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Prevotella intermedia* are associated with dental plaque and periodontal diseases.

5.1 Minimal inhibitory concentration (MIC) of PS/CHX for dental caries associated bacteria

The minimum inhibitory concentrations (MICs) of PS and CHX alone and in combination for *S. mutans* and *S. sobrinus* were determined using a broth microdilution assay in 96-well microtiter plate (Amsterdam, 1996). *S. mutans* and *S. sobrinus* were grown overnight at 37°C under anaerobic conditions in Todd-Hewitt broth containing 0.3% yeast extract (THYE) broth supplemented with 0.01% hog gastric mucin (pH 7.0). The overnight growth was diluted to approximately 10⁵ CFU/ml. PS (200 to 0.195 µg/ml) and CHX (50 to 0.098 to µg/ml) alone and in combination were serially diluted in THYE (100 µl), and 100 µl of bacterial suspension was added to each well. Plates were incubated at 37°C for 24 h and read at 600 nm using a microtiter plate reader (Multiskan Ascent, Labsystems, Helsinki, Finland). The MIC was taken to be the lowest concentration of antimicrobial that completely inhibited growth. The MIC for the combination of PS and CHX was found to be significantly lower than the MIC for either of PS and CHX alone, and the combination of PS and CHX was found to be synergistic in the inhibition of microbial growth for *Streptococcus* spp tested (**Table 5**).

Table 5: MIC (µg/ml) of PS and CHX alone and the PS/CHX combination for dental caries associated bacteria

Organism	MIC (µg/ml)		
	PS	CHX	PS/CHX
<i>S. mutans</i> UA 159	> 200	6.25	3.12/0.78
<i>S. sobrinus</i>	> 200	1.56	6.25/1.56

5.2 Effect of PS/CHX on biofilm formation in dental plaque and caries associated bacteria

Biofilms were assayed using a modified quantitative biofilm assay method (Jackson, *et al.*, 2002). The overnight cultures of *S. mutans* and *A. naeslundii* were diluted to 1% in 4 x dilute THYE at pH 7.0 and Tryptic Soy broth supplemented with 0.3% yeast extract (TSBYK, plus hemin & menadione), respectively. Biofilms of bacteria were grown at 37°C, in an anaerobic chamber (5% CO₂) in a 12-well tissue culture polystyrene plate (Corning Inc., New York). Aqueous solutions of PS and CHX were prepared separately and appropriate volumes of each were added to 12-well plates individually and in combination. The total volume of each well was made up to 2 ml with the 1% inoculum. The wells without antimicrobials served as controls. After 20 h incubation, the medium containing planktonic cells in each well were removed and biofilm was rinsed with PBS. After adding 2 ml of PBS to each well, the plate was sonicated for 15 seconds and dislodged biofilm was mixed well with the pipette tip. Further, the suspension from each well was serially diluted (10-fold dilution) and plated 100 µl of each dilution on THYE agar and Columbia Blood Agar, respectively. The plates were incubated at 37°C, anaerobically for 48 h and colonies were counted. Plates from the wells treated with PS and CHX in combination contained significantly fewer colonies than either treatment on its own. Significantly lower concentrations of PS and CHX were required for an equivalent number of colonies formed, as compared to PS or CHX treatment alone. The combination of PS and CHX was synergistic in inhibiting the growth of biofilm embedded *S. mutans* and *A. naeslundii* (**Figs. 5 and 6**).

Fig. 5: Effect of PS and CHX alone and in combination on biofilm formation in *Streptococcus mutans*

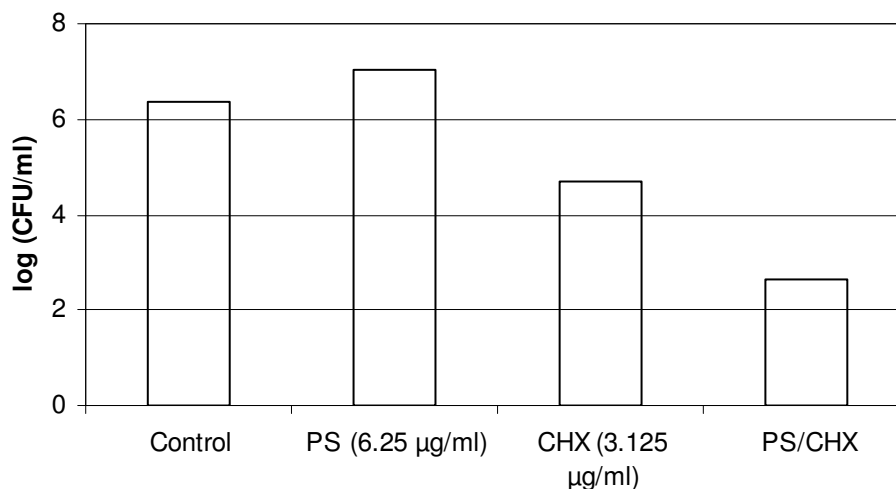
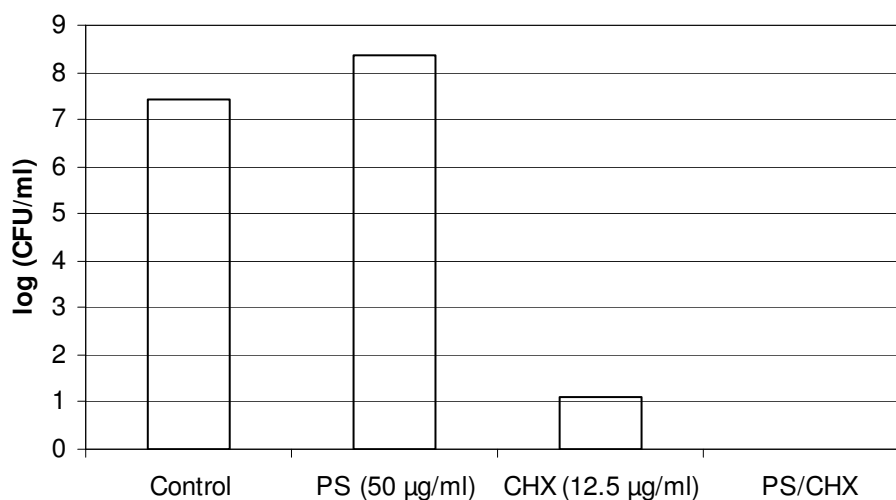


Fig. 6: Effect of PS and CHX alone and in combination on biofilm formation in *Actinomyces naeslundii*



5.3 Effect of PS/CHX on biofilm formation in periodontal disease associated bacteria

In vitro microplate assays were performed to determine the synergistic effect of protamine sulfate and chlorhexidine combination on the growth of biofilm forming periodontal diseases-associated bacteria such as *Prevotella intermedia* and *Porphyromonas gingivalis*. Overnight cultures of each

bacterial strain were grown in 25 ml Todd Hewitt (TH) broth supplemented with menadione and hemin as described previously (Davey, 2006) under anaerobic conditions at 37°C for 24 hrs. Control (water) and 2 fold dilutions of combo were added, 20 µl/well. A biofilm media was prepared (modified salt base plus bovine serum albumin (BSA), α-ketoglutarate, tryptone, menadione and hemin) as described previously (Milner et al., 1996) diluting overnight culture 1:10 and aliquoted into wells (180 µl/well). The 96 well plates were incubated under anaerobic conditions at 37°C for 48 h and were read at 600 nm using a microtiter plate reader (Labsystems, Multiskan Ascent, Helsinki, Finland). The media was removed and the plate washed once with sterile distilled water, air dried for 1 hour, stained for 15 minutes with crystal violet, stain removed and rinsed twice with sterile distilled water, air dried and then crystal violet was solubilized in 33% acidic acid solution and the plate was read at 630 nm. The combination of protamine sulfate and chlorhexidine inhibited biofilm formation in both *P. intermedia* and *P. gingivalis* with an appreciable synergy against the former (**Figs. 7 and 8**).

Fig. 7: Effect of PS/CHX combination on biofilm formation in *Prevotella intermedia*

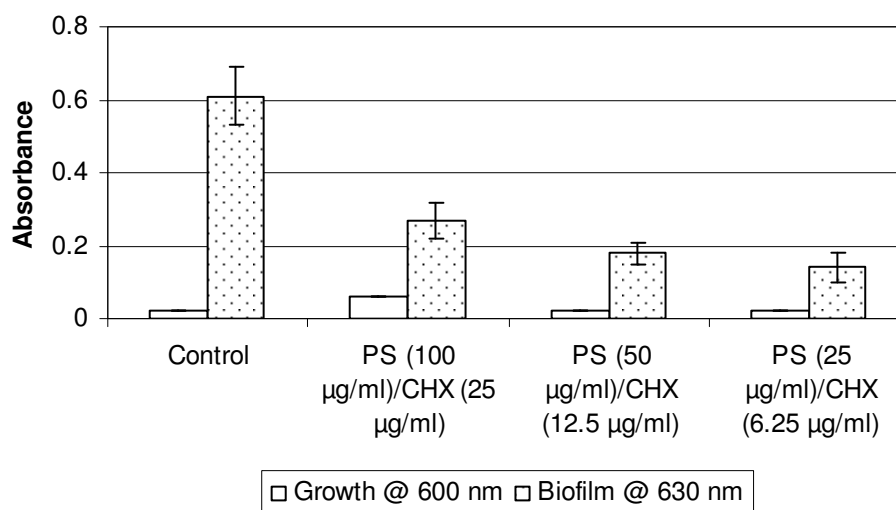
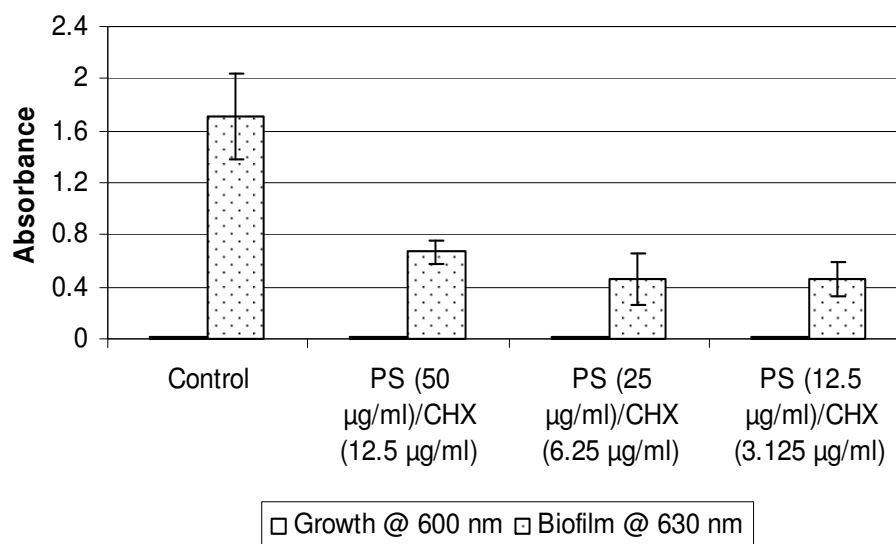


Fig. 8: Effect of PS/CHX combination on biofilm formation of *Porphyromonas gingivalis*

6. Safety

6.1 Protamine Sulfate

6.1.1 Chemistry

Protamine sulfate is a cationic polypeptide salt (CAS# 53597-25-4, Synonyms: Salmine sulfate) with the amino acid sequence “mprrrrsssrvrrrrrrrrrrgrrrr” (NCBI accession # P69014).

6.1.2 Pharmacology

Neutralization of heparin occurs within 5 min after intravenous administration of an appropriate dose of protamine sulfate. Although the metabolic fate of the heparin-protamine complex has not been elucidated, it has been postulated that protamine sulfate in the heparin-protamine complex may be partially metabolized or may be attacked by fibrinolysin, thus freeing heparin.

6.1.3 Toxicology

The median lethal dose (LD50) of protamine sulfate for both rabbits and mice was found to be between 200 mg/kg and 300 mg/kg when given subcutaneously.

6.2 Chlorhexidine

6.2.1 Chemistry

Chlorhexidine is a bis-guanide with the Molecular Formula $C_{22}H_{30}Cl_2N_{10}$, Melting point $134^{\circ}C$, Mol. Wt: 505.46 and CAS# 55-56-1 for chlorhexidine base.

6.2.2 Pharmacology

Studies conducted on human subjects and animals demonstrate that chlorhexidine gluconate is poorly absorbed from the gastrointestinal tract. The mean plasma level of chlorhexidine gluconate reached a peak of $0.206\mu g/g$ in humans 30 min after they ingested a 300 mg dose of the drug. Detectable levels of chlorhexidine gluconate were not present in the plasma of these subjects 12 hours after the compound was administered. Excretion of chlorhexidine gluconate occurred primarily through the faeces (~90%). Less than 1% of the chlorhexidine gluconate ingested by these subjects was excreted by the urine.

6.2.3 Toxicology

The lethal dose (LD50) of chlorhexidine for mice was found to be 2515 mg/kg oral, 44 mg/kg intraperitoneal, 632 mg/kg subcutaneous, 24 mg/kg intravenous administration

7. Risk Analysis

Although PS/CHX combination is not used in any of the currently marketed medical devices, we do not anticipate any risks of safety due to the following reasons:

- a) There are no known interactions of protamine sulfate with chlorhexidine.
- b) Protamine sulfate has been successfully used as intravenous injections for almost 50 years. Allergic reactions associated with administration of PS have been reported but the patients developing adverse reactions had undergone major surgeries, a clear connection to PS administration was not obvious because of the surgery-related haemodynamic reactions (Lindblad, 1989).
- c) Chlorhexidine in combination with silver sulfadiazine is successfully used for coating central venous catheters (Arrow International, PA, USA) (Sampath, et al., 2001). Arrow International reports that no case of hypersensitivity has been reported in the US after the introduction of CHX containing central venous catheter product ARROWg+ard Blue in 1990. ARROWg+ard Blue central venous catheters have an external surface coated with chlorhexidine acetate and silver sulfadiazine. In addition, as of October 1999, there have

been no reports of any unanticipated adverse device events, nor are there any reports of anaphylaxis/hypersensitivity to ARROWg+ard Blue Plus antimicrobial catheters. Compared to original ARROWg+ard Blue catheters, ARROWg+ard Plus external surface treatment represents a three-fold increase in the amount of chlorhexidine acetate and unchanged amount of silver sulfadiazine. The average amount of chlorhexidine, silver and sulfadiazine applied to the external surface of the catheter is 425 µg/cm, 24 µg/cm, and 56 µg/cm, respectively. The average amount of chlorhexidine applied to the catheter body and extension line internal lumen is 22 µg/cm.

- d) The biocompatibility studies done on PerioGard (Colgate Oral Pharmaceuticals, Inc. Lakewood, NJ) containing 0.12 % CHX, shows that it is neither carcinogenic nor mutagenic and it had no adverse effect on the pregnancy.
- e) Chlorhexidine resistance was reported in *Providencia stuartii*, *Pseudomonas aeruginosa* and *Proteus mirabilis* due to an excessive use of chlorhexidine in hospitals. In resistant strains, cell wall becomes impermeable to chlorhexidine (Stickler, 2002). However, PS/CHX combination, PS alters the permeability of microbial cell membrane to facilitate the transport of antimicrobial agent to the cytoplasm. Protamine sulphate increases the membrane permeability by punching holes and dilating ion channels as well. Thus, PS in combination with CHX would not likely develop bacterial resistance to CHX due to cell membrane becoming impermeable. Interestingly, a multicenter clinical trial involving a total of 94 periodontitis patients, conducted by Colgate Oral Pharmaceuticals, Inc (Lakewood, NJ) showed no change in oral microbial ecosystem and bacterial resistance after six-month use of PerioGard containing 0.12% chlorhexidine gluconate as oral rinse (Persson, *et al.*, 1995). Sampath, *et al* (2001) reported that there is very low risk of developing antimicrobial resistance due to long term use of chlorhexidine and silver sulfadiazine-impregnated catheters (ARROWg+ard Blue catheters)
- f) *E. coli* that expresses OmpT protease enzyme to degrade antimicrobial peptides as one of the virulence mechanisms has been reported to show resistance to protamine sulfate (Stumpe, *et al.*, 1998). However, a recent *in vitro* study has shown the synergistic inhibitory effect of PS/CHX combination on *E. coli* growing in biofilms (Darouiche *et al.*, 2007). To reduce the effective concentration of an antimicrobial agent and also to minimize the risk of developing resistance, toxicity or side effects, combinations of

antimicrobials are preferred over a single agent for coating catheters (Kim, *et al.*, 2002). Thus, the benefits of using PS and CHX combination with both antimicrobial and antibiofilm activities outweigh the possible safety risks.

8. Desirable features

- 8.1 PS/CHX combination is a broad-spectrum synergistic antimicrobial composition with antibiofilm activity
- 8.2 PS in combination with CHX is required in quantities appropriate for human use
- 8.3 Both PS and CHX are FDA-approved compounds for human use
- 8.4 Currently marketed antimicrobial central venous catheters (ARROWg+ard Blue catheters, ARROWg+ard Plus) already contain CHX in combination with silver-sulfadiazine
- 8.5 This combination of two non-antibiotic compounds is unlikely to pose any safety or bacterial resistance concerns.

9. Applications

- 9.1 Coating medical devices such as urinary catheters, central venous catheters, cerebrospinal fluid shunts, peritoneal dialysis catheters, airway management devices (e.g. endotracheal tube), vascular grafts, intraocular lenses, prosthetic cardiac valves, cardiac pace makers, and prosthetic joints.
- 9.2 Wound care products
- 9.3 Oral care products
- 9.4 Animal health care products
- 9.5 Disinfectants for food & beverage and dairy industries

10. References

10.1 Patents

Madhyastha, S. 2007. Antimicrobial compositions and uses thereof. U.S. Patent Publication No. 20070003538 A1; PCT International Publication No. WO/2007003028 A1.

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- Madhyastha, S.** December 2006. Synergistic antimicrobial compositions and methods for reducing biofilm formation. U.S. Patent No. 7,144,992.
- Madhyastha, S.** March 2005. Synergistic antimicrobial compositions and methods of inhibiting biofilm formation. U.S. Patent No. 7,314,857; Canadian Patent No. 2452032.

10.2 Publications

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