

May 9<sup>th</sup>, 2000

Mr. Steven Frank  
Senior Scientist  
Klearsen Corporation  
11192 Twin Spruce Rd.  
Golden, CO 80403

Dear Mr. Frank,

Please find enclosed the final report for the testing conducted by Bacterin Inc.

#### Introduction

Bacteria that attach to surfaces and form hydrated polymer matrices of their own synthesis are called biofilms. Formation of these bacterial communities and their inherent resistance to antimicrobial agents are at the root of many persistent and chronic bacterial device related infections. Bacterin's testing methods invoke a biofilm phenotype and are considered "state-of-the-art" testing methods for anti-infective medical device coatings. The biofilm model, to date, has been an excellent clinical predictor in anti-infective coating research.

#### Methods and Procedures

In accordance with the FDA's guidance for conventional and antimicrobial Foley catheters, a clinical isolate of *Staphylococcus aureus* was tested on the finished form of a pure silver wire. The isolates were maintained in a chemostat at  $10^6$  throughout the trial period to insure a consistent challenge. Dilutions of *Staphylococcus aureus* were made to reflect a  $10^3$  challenge on a daily basis. The adhesion process was observed in a one-pass flow cell bioreactor under highly standardized conditions. Bacterial adherence and viability are determined by; scraping and plating of the colonized silver, and direct microscopic observation using Molecular Probes BacLight Live-Dead stain. Bacterial challenge was provided by a modified syringe pump at a continuous rate of 10mL/day.

A modified infusion pump was used to conduct the study. The infusion pump utilized 4 10mL syringes simultaneously inoculating 4 bioreactors. 5 mm<sup>2</sup> was scraped with a sterile scalpel, sonicated for 1 minute, and vortexed for 30 seconds in 100 uL of phosphate buffered solution (PBS). The PBS was then plated on nutrient agar and incubated for 24 hrs to determine growth in cfu/mL. Another 5 mm<sup>2</sup> segment was stained

with Molecular Probes BacLight Live/Dead stain to determine cell viability. Cell attachment and viability was determined by direct epi-fluorescent microscopic examination. Measurement parameters were in % area of coverage, and % of live cells vs. dead cells. In accordance with standards traceable to NIST standards, 20 random fields were examined.

### **Application of the Klearsen Active Antisepsis**

Each flow cell was modified to allow the Klearsen Active Antisepsis control system to contact the test coupon. The return path for the small current flow was through the ionically rich PBS. The control system was connected to the test coupon and the return path connection was made to a stainless steel port which also served to introduce the flow into the cell.

The Active Antisepsis control system provided a pre-set current through the flow cell that was independent of the system impedance. The current was selected to achieve the desired surface current density. The control system was applied to cells 2, 3 and 5 for 15 minutes.

#### **Flow Cell #1            Material Control**

Flow cell #1 was the material control sample offering no activation by the Klearsen Active Antisepsis control system. The challenge was applied for 48 hours. The coupon was then treated according to the staining and plating protocol to determine colony counts and to image the percentage coverage.

#### **Flow Cell #2            Show Kill**

Flow cell #2 was intended to demonstrate a single application of the Klearsen Active Antisepsis control system after a biofilm had grown on a significant portion of the surface. This cell was for direct comparison to cell #1. The challenge was applied for 48 hours. The coupon was then treated according to the staining and plating protocol to determine colony counts and to image the percentage coverage.

#### **Flow Cell #3            Kill through a dead layer**

Since a layer of dead bacterial cells can render a surface-treated coupon defenseless to re-growth, it was the intention of cell #3 to test the ability of the Klearsen Active Antisepsis to penetrate a dead layer of cells and kill new growth. This cell saw the standard 48 hour challenge followed by the application of the Active Antisepsis control system to kill the developed layer. Subsequently, a new challenge for 48 hours added additional cells over the dead layer. After the second 48 hour challenge, the Klearsen Active Antisepsis

control system was activated and the coupon was then treated according to the staining and plating protocol to determine colony counts and to image the percentage coverage.

Flow Cell #4                    **Continuous treatment**

Flow cell #4 allows us to see the effect of a continuous administration of the active antiseptics system. Here the Active Antiseptics control system was activated during the entire 48 hour challenge. It was operated at a level approximately ¼ of that used for the single application kill. The challenge was applied for 48 hours. The coupon was then treated according to the staining and plating protocol to determine colony counts and to image the percentage coverage.

Flow Cell #5                    **Current Control**

In order to rule out the effects of current alone causing any bactericidal effects, Flow cell #5 was added. This cell used a Stainless Steel coupon and was energized with the Klearsen Active Antiseptics control system in the same manner as Flow cell #2. The challenge was applied for 48 hours. The coupon was then treated according to the staining and plating protocol to determine colony counts and to image the percentage coverage.

Results:

Plate counts from 4.22 ( 48 hours after inoculation)

Flow cell # and conditions	Dilution -0	Dilution -1
#1, material control	TNTC	>300
#2, charge 15 min 100 uA	175	54
#3, re-growing of biofilm after first charge	13	0
#4, permanent charge 29uA 48 h	0	0
#5, current control, stainless steel	>150	41

Confocal microscopy from 4.24

Flow cell # and conditions	surface colonization	killed cells
#1, material control	45-55%	50%
#2, charge 15 min 100 uA	45-55%	70%
#3, re-growing of biofilm after first charge	65%	>95%
#4, permanent charge 29uA 48 h	45-55%	80%
#5, current control, stainless steel	95%	80-90%

Conclusion and Recommendation:

Based on our initial testing, the Klearsen Corp. technology is superior to all commercially available anti-infective coating technologies. The need for long term medical device implants to have a continuously renewable source of eluting antimicrobial compound appears to be met by this technology. Further testing, both in-vitro, and in-vivo, are needed to elucidate the benefits of what is clearly a superior technology in this testing model.

Sincerely,

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