# “THE KILLING AND SOLUBILIZING EFFECTS OF SOLUTION A ON HOMOGENEOUS AND HETEROGENEOUS PLANKTONIC ORGANISMS, SESSILE ORGANISMS, BIOFILM ORGANISMS, AND BIOFILM STRUCTURES”

Studies Conducted by:
BEIERLE LAB TECHNOLOGIES
JOHN W. BEIERLE, PhD - CONSULTANT

## Studies to Prove the Effectiveness of EXXCL Oral Blue Antimicrobial Technology in the Complete Destruction and Solubilization of Biofilm Organisms and Structures

***Introduction to the problem:*** Biofilms have been recognized as moderate to heavy aggregated masses of bacteria for over a hundred years. The "biomats" coating on sand filters of drinking water supplies were noted in Germany in the 1800's. Back-flushing techniques were developed to disrupt those "Schmutzdeke" which were known to reduce flow rates of water supplies. Very slowly, mankind began recognizing biofilms in their numerous varieties and forms. Recognition of a problem is the first step in understanding the intricacies of any problem's complexity.

Eventually recognition, discovery and problem solving began. This is what is occurring in the world of biofilms. The intricate interactions of microbes within the biomass allow for communication between single celled creatures, which exposed the dogma of simplicity relating to bacterial interactions. Controlling biofilms is now a recognized major problem. To address the biofilm problem, we utilized Solution A, a proprietary mixture with remarkable antimicrobial properties. These properties extend beyond killing free floating or planktonic cells and indeed have disruptive killing and even solubilizing effects on the sessile cells within biofilms.

The studies described here were defined with both single homotypic cells as well as mixture of multiple or heterotypic cell types under a variety of conditions.

## *Materials and Methods*

Oral bacteria were obtained from clinical isolates and never used past the seventh passage. Other bacteria were obtained from the American Type Culture Collection (Virginia). Oral flora was obtained from patients with heavy plaque and/or periodontitis. Samples were obtained from plaque, saliva and subgingival sources. No attempt was made to discriminate each individual species. The cultures were grown in reduced air conditions, but not anaerobically.

Bacteria were grown at room temperature at 35°–37° C as indicated. Bacteria were grown in an enriched media of:

| | | |
|---|---|---|
| Brain-Heart Infusion | 2 g | |
| Todd-Hewitt Broth | 2 g | per liter |
| Yeast Extract | 2 g | deionized water |
| Dextrose | 10 g | |
| Nutrient Broth | 4 g | |

Agar was added to 0.8% for soft agar preparation to count colonies in pour plates by cfu assessments. Media was obtained from BBL, Cockeysville, MD. Bacteria were inoculated by syringe into stomach catheter tubing until the tube was filled and then placed in petri dishes submerged in sterile media. The inoculating dose was ca. $10^6$. In some experiments, bacteria were inoculated directly into Falcon #1007 positively charged tissue culture dishes (Falcon Plastics, Oxnard, CA). Inoculum were taken from cultures grown to mid-log phase of growth; 18-24 hours culture. Media was changed in tubing twice daily and every 24 hours in petri plates.

Biofilms were allowed to progress for 1-3 or more days depending on the experimental protocol. Incubation temperatures varied depending upon experimental conditions as well. Biofilms made in tubing were utilized in the scanning electron microscope studies. Petri plate biofilms were examined visually or by low magnification microscopy and were photographed with a Minolta X-7000 camera fitted with an AF-50 macro lens. All biofilms were washed with sterile saline to remove planktonic cells prior to testing with Solution A. Tubing was sliced in half lengthwise to expose the biofilm to washing and SEM observation. Tubing preparations were sprayed with platinum-iridium metallic spray under vacuum twice to ensure adequate coating on curved surfaces. The concentration of solution A started at 1:5.

## *Results*

Petri dish biofilms were made up of homogeneous, and heterogeneous, mixed oral flora. The positively charged surface of these dishes allowed for increased adherence of negatively charged bacterial cells. Biofilm buildup increased day by day and by the end of two days (24 hours), an extensive mass was noted. A 20 minute exposure of 1:5 Solution A to the two day mixed culture biomass showed an extensive breakup of the adherent mixed culture mass (photo 1). When the petri dish was rinsed to remove debris (photos 2, 3,4) it became obvious that the disruptive action of Solution A is extremely extensive.

Debris was centrifuged into pellet (x 5000g) and resuspended in 12 ml media in a test tube. The tubes were incubated for five days and no growth was detected demonstrating that complete destruction of all living forms occurred along with destruction and solubilization of the biofilm masses of extracellular material (ECM) in which the bacteria were imbedded.

### ***Summary Statement***

These studies revealed that Solution A rapidly and effectively demonstrated its complete destructive capacity for the disruption of the ECM holding the mass together, and with the kill of the bacteria within that mass of gelatinous material. These studies revealed that Solution A rapidly and effectively demonstrated its complete destructive capacity of the ECM holding the mass together, and the kill of the bacteria within that mass of gelatinous material. Cultures derived from human sources (mixed oral flora) do not form petri dish biofilms well at room temperatures, but do so at 35°-37°C which is their natural temperature (photo 18).. The temperature for initial adherence and growth is very important in biofilm formation and maturation.

Solution A is extremely effective in biofilm destruction on petri plates that incubated at 30 or 45 minutes in Solution A (photos 20 and 19). What is striking is that Solution A disrupts biofilms so completely and almost simultaneously kills the embedded microbes.

Biofilm destruction starts as early as 10 minutes and further breaks down with each successive minute thereafter (photo 20 and 25). The younger the biofilm, the more readily susceptible to the actions of Solution A. However, even old, extremely dense biofilms, were noted for destruction. At 24 hours exposure to Solution A., absolute cleanliness was demonstrated.

***Experiments with Biofilms Grown in Stomach Catheter Tubing:*** Our previous experiments have shown us that homogeneous cell population derived biofilms are quite different than heterogeneous cell populations and mixed oral flora populations were found to be

the toughest and densest biofilms. Therefore, I challenged Solution A to the most difficult biofilm I could create.

Single cell population biofilms, with the exception of Candida albicans-derived masses, were noted to be much easier to disrupt and destroy than heterogeneous populations of bacteria comprising biofilms. Our general view made was that if we could destroy complex heterogeneous biofilms, we ought to be able to destroy, more easily, a homogeneous biofilm.

In our experiments with catheter tubing biofilm, we cut the tubing lengthwise, to view the internal composition of the tubing by scanning electron microscopy. We performed a time course study on biofilm buildup with mixed oral flora containing rods, spirochetes, cocci and diphtheroids and examined the effects of Solution A on tubing biofilm of different levels of maturation. During the first 24 hours, an incomplete, non-confluent biofilm occurred. More adherent rods were noted than cocci (photo A).

Addition of Solution A at room temperature to cells grown at 35° for 24 hours showed extensive break-up of both cells and biofilm matrix in as little as 15 minutes (photo B). All pictures were taken at 1,200x magnification. The destruction of total biofilm is so extensive that only a few globules of material are left after as little as two hours. Collecting debris by centrifugation and resuspending the disrupted cell-matrix pellet in (1) enriched media and (2) media containing purple broth base was performed for growth analysis to detect any viability. No growth was noted even after 10 days incubation in either media. The purple broth based color indicator media is so sensitive that as little as 100 microbes initial inoculum would be detected in as little as 12 hours, whereas no growth was detected after 250 hours incubation. Photo C shows 30

minute breakdown continuing and photo D represents two hour complete destruction of biofilm and totally clean tubing.

***Relation to Dr. Wiliam Costerton's Report:*** Both of our results showed virtual instantaneous kill of the sessile cells. Dr. Costerton showed that slime enclosed aggregates of E. coli roll up into compact aggregates and stain as dead cells by confocal microscopy. We find a similar rolling up effect in petri dish cultures. Holes start "punching in" to the center and edges of the biofilm matrix and the biofilm starts detaching and rolling up. When we detach biofilms and disperse the matrix by agitation, we do not detect growth when this biofilm debris is inoculated into fresh medium. Corresponding studies are found in Dr. Costerton's Figures 1-8. Dr. Costerton found the basic same kill results using Klebsiella proteus, E. coli, Pseudomonas and Enterobacter, and Staph aureus. We have examined homogeneous cell population biofilms using E. coli, Staph, and Pseudomonas and found identical results as Costerton's group.

We have found, however, the multiple species biofilms are harder to destroy. Yet, our inoculum of mixed oral flora, even in cultures up to two weeks old, which are tenacious and very dense, are still rapidly killed by Solution A. Actual dissolution of the biofilm occurs thereafter and with additional time, e.g. 12- 24 hours, even the most tenacious biofilms are solubilized, (photo 24).

With regard to suspension cells, both our lab and Dr. Costerton's lab find destruction of these planktonic cells to be a virtual instantaneous process. The Costerton group found Pseudomonas biofilms difficult to kill with Solution A in an instantaneous manner, but kill was still extensive. Our own studies have confirmed that extended exposure of Solution A has provided kill and destruction of various resistant biofilm when longer exposure to Solution A is provided, e.g. 12-

24 hours. Dr. Costerton suggested that if the resistant biofilms were exposed to solution A for longer time periods, he suspected that the microbes would probably all be killed. For example, 2-3 week biofilm buildup, by Candida albicans at room temperature, provides a resistant biofilm, which requires a 12-24 hour exposure to Solution A for complete destruction.

## *Conclusions*

Solution A is effective in killing both sessile and planktonic cells rapidly and effectively. Furthermore, Solution A kills cells within the biofilm mass and destroys the biofilm extracellular matrix both rapidly and efficiently. The killing, solubilzing effect destroys heterogeneous bacterial populations as well as homogeneous cell populations. The biofilms used in our experiments were made on stomach catheter tubing and also on positively charged petri plates because positively charged plates assured tight binding..

Dr. Costerton's group found similar, if not identical findings on biofilms formed in reaction chambers where cells growing in suspension in clumps (detached biofilms) were also considered biofilms matrices, similar to surface attached cells. In earlier reports, we had previously formed aggregates of cells by rotary mediated shaker experiments using suspension cultures. These clumps were similar to Costerton's suspension clumps. In our experiments, we found rapid kill and dispersion of these aggregates. We also noted the cessation of binding of microbes in the presence of Solution A, to solid surfaces as suggested by Dr. Costerton in the cleaning of tooth surfaces and pipes. We therefore suggest that Solution A has adhesion inhibitory properties as well as dispersal properties.

Dr. Costerton also stated that there is a paucity of agents capable of even partial cleaning of biofilms; something which we have also noted. Solution A, on the other hand, has provided both our labs with an excellent agent capable of performing the job on virtually every bacteria biofilm we have tested.

Dr. Costerton's biofilm generating capacities with his chemostat has provided him with an excellent procedure for biofilm production and testing. We on the other hand, used catheter tubing and positively charged petri plates. Both of these approaches with similar findings provide a strong body of evidence leading to the same results of Solution A. The Costerton group's approach with confocal microscopy and vital staining also parallels our own finding with scanning electron microscopy and measurements of actual cell growth and kill by way of colony forming unit measurement.

Solution A is a uniquely effective means of biofilm kill and degradation on homogeneous and heterogeneous sessile biofilm organisms and biofilm structures.