Wound Healing and Biofilms
Clinical Relevance and Therapies

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Pre-Test on Biofilms and Wound Infections

Which one of the following statements about planktonic and biofilm bacteria is FALSE?

1. Most bacterial species and strains can grow as both planktonic and biofilm phenotypes.
2. Bacteria in a biofilm community are very tolerant to most antibodies, antibiotics and antiseptics that rapidly kill the bacteria when they are growing as planktonic bacteria.
3. Only a small percentage of chronic skin wounds contain biofilm communities.
4. Infections cause chronic inflammation and impair healing in other diseases including periodontal disease, osteomyelitis, cystic fibrosis and Crohn’s disease.

Basic Background of Bacterial Biofilms

• Planktonic bacteria – single, non-attached bacteria
• Biofilm bacteria – a structured community of bacteria cells enclosed in a self-produced exopolymeric matrix this is tightly adherent (sessile) to living or inert surface
• Quorum sensing – process by which bacteria molecules shift growth from planktonic to biofilm phenotypes
• Biofilms provide a protected mode of growth – evolutionary defense against natural predators: bacterial viruses, amoeba, and microbicides. Also protects against phagocytosis (inflammatory cells), antibodies, natural reactive oxygen species (ROS), antibiotics, antiseptics, and disinfectants
• Exopolymeric matrix of biofilms consists of predominately of polysaccharides along with bacterial DNA and proteins that are extremely inflammatory to innate and acquired immune systems
• Persistor bacteria are quiescent (not metabolically active) and are not killed by antibiotics that only act on metabolically active bacteria

Biofilms Identified in 60% of Biopsies of Chronic Wounds but in Only 6% of Acute Wounds

Confocal laser scanning microscopy (top view) of (A) planktonic Pseudomonas aeruginosa, (B) biofilm community. (C) Schematic representation of polymicrobial bacterial biofilm formation (side view).


Clinical isolates of *S. pneumoniae* produce different amounts of EPS biofilm. CLSM images of biofilm development by clinical isolates of *Streptococcus pneumoniae* stained with BacLight after 6 days of culture showing viable (green fluorescence) and nonviable (red fluorescence) pneumococci within the biofilms. Images are maximum projections or reconstructed confocal stacks consisting of a series of *x* and *z* sections. Sideviews (*YZ* – left and *XZ* – bottom) are sagittal sections of the biofilm. Scale bar = 30 μm.

* Hall-Stoodley et al. BMC Microbiology 2008, 8:173

**More EPS Requires Higher Concentrations of Antibiotic to Inhibit Growth**

<table>
<thead>
<tr>
<th>S. pneumoniae isolate</th>
<th>Serotype</th>
<th>Planktonic IC₅₀ (μg/ml)</th>
<th>Biofilm IC₅₀ (μg/ml)</th>
<th>Fold Increase</th>
<th>Number of replicates</th>
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<tbody>
<tr>
<td>BS 68</td>
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<tr>
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<td>&lt;2</td>
<td>63</td>
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*Highest concentration of azithromycin producing no turbidity after overnight incubation with antibiotic with planktonic cells. Turbidity was scored by eye as well as by determining the highest antibiotic concentration that was within the standard deviation of the optical density measurements of 6--8 replicate untreated controls (no antibiotic).

†Indicates high ranked BFI strain.

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**Biofilm Exopolymeric Matrix Contains Substantial Amounts of Bacterial DNA**

DNA staining of EPS matrix of *S. pneumoniae* and disruption with DNase. Panel A. Biofilms stained with ProGreen, which stains double stranded dsDNA intensely green. Panel B. Biofilms stained with SYTO9, which stains intranucleic acid red, shows that *S. pneumoniae* biofilms treated with 100 μg/ml Pulmozyme (+) a synthetic Pulmozyme (β-glucanase) cleaved DNA which is widely used in biofilm research. DNA was visualized with 1000 μg/ml Pulmozyme and then treated with 100 μg/ml DNase I (Genzyme), were substantially reduced compared to untreated biofilm in both high and low BFI strains. Scale bar = 20 μm.

**Panel B.** Quantification of reduction in biofilm volume (total biomass) measured by COMSTAT 2 algorithm treatment with different concentrations of Pulmozyme (β-glucanase) Control: 0 μg/ml Pulmozyme (−). Bacterial CFU counts were taken in triplicate and at least 1000 erythrocyte-inoculated cells were counted in each experiment. Error bars represent standard error of the mean: n = 10. Statistical comparisons were made using Student’s *t* test (Table 1). 10 μg/ml Pulmozyme was a substantial but not significant difference from untreated control (Pulmozyme = 0.05; *P* value = 0.01). Statistical comparisons were made using Student’s *t* test (Table 1). Scale bar = 20 μm.

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TEM of Staph aureus Biofilm Growing on Rabbit Femur


Question: Does formation of biofilm colonies in a wound retard healing?

Answer: YES or NO

Biofilm Formation by Staphylococcal Species Delays Healing of Mouse Cutaneous Wounds

Schierle et al., Wound Rep Reg 17:354, 2009

How Does The Immunological Response to Biofilms Cause Tissue Damage?

In Panel A, planktonic bacteria can be cleared by antibodies, phagocytosis, and are susceptible to antibiotics. Adherent bacterial cells (Panel B) form biofilms preferentially on inert surfaces or devitalized tissue, and these sessile communities are resistant to antibodies, phagocytosis and antibiotics. Phagocytes (Panel C) are attracted to the biofilms, but phagocytosis is frustrated. Phagocytic cells still release enzymes and ROS. Phagocytic enzymes (Panel D) damage tissue around the biofilm, and planktonic bacteria are released from the biofilm, causing dissemination and acute infection in neighboring tissue.

Costerton, Stewart, Greenberg, Science 284, 1999

Question: Do all antimicrobical wound dressings effectively kill biofilm colonies grown on pig skin explants?

Answer: YES or NO

Can Dressings Disrupt & Kill Mature Biofilms?

1. Effects of Dressings on Mature P. aeruginosa Biofilms After 1 and 3 Days of Exposure

2. 24 hr Continuous Exposure of Mature PAO1 Biofilm on Porcine Explants

Question: Why are bacteria in biofilms hard to kill?

Which answers are TRUE?

1. Exopolymeric matrix reacts with antiseptic molecules and reduces diffusion of large microbialid molecules
2. Persistor bacteria with low metabolic activity are not sensitive to antibiotics that only kill growing bacteria
3. Oxygen diffusion is limited which promotes growth of anaerobic bacteria
4. Different bacteria synergistically secrete enzymes (catalyase) that destroy ROS and bind antibiotics (MRSA)
5. All the above are true statements

Antibiotics verses Biofilms on Inert Surface

Biocides verses Biofilms
Bacteria in Biofilms are Hard to Kill

After 60 minutes of exposure to dilute bleach (Dakin's solution), many bacteria in this biofilm were dying (green cells), but many cells in the interior of the biofilm were still alive (orange cells) Costerton, Sci Am, 2001

Reaction-Diffusion Problem

Hypochlorous acid rapidly reacts with molecules that form the biofilm exopolymeric matrix, which limits its diffusion into the center of the biofilm colony. Phil Stewart, MSU Center for Biofilm Engineering

Topical Antibiotics Effectively Kill Planktonic Bacteria in Pig Skin Wounds But Only Reduce Bacterial in Biofilms 2-Logs After 48 Hours

Tobramycin rapidly kills planktonic Pseudomonas aeruginosa ( ), but is not effective against biofilm Pseudomonas ( ).


Davis et al., Wound Rep Regen 16:23-29, 2008
Metabolic Activity of Pseudomonas aeruginosa in Mature Biofilms is Limited to the Surface Layers

- Only fluorescent bacteria are metabolically active
- Only located in outer layers of the biofilm matrix
- Antibiotics only kill metabolically active bacteria

Phil Stewart, Montana State University Center for Biofilm Engineering

Distribution of Aerotolerance of Bacterial Populations in Chronic Wounds

Figure 1: Distribution of Bacterial Populations in Chronic Wounds in Relation to Aerotolerance. Diabetic, venous, or pressure ulcer types were analyzed separately using pyrosequencing and the resulting populations grouped into 3 categories based upon their suggested aerotolerance. This figure graphically illustrates the relative distribution of these functional categories among the wound types.


Principles of Biofilm Based Wound Care

1. Frequent debridement of wounds to physically remove biofilm communities
2. Use of an effective bacterial barrier dressing after debridement to prevent reformation of biofilms
3. DNA based identification of bacterial species in wounds – personalized topical antimicrobials
4. Alter topical & systemic antimicrobial treatments to prevent emergence of dominant bacteria from polymicrobial populations


Wound Slough Harbors Bacterial Biofilms

Biofilm-Based Wound Care

Dissolved Oxygen Gradients Measured in Biofilm

Z. Lewandowski, D. De Beer, P. Stoodley
**Question:** How quickly can planktonic bacteria form protective biofilms in wounds after debridement?

Which answer is true?

1. 7 days
2. 5 days
3. 3 days
4. 1 day

**Biofilm Maturity Studies Indicate Sharp Debridement Opens a Time-Dependent Therapeutic Window**

<table>
<thead>
<tr>
<th>Days</th>
<th>Mature</th>
<th>1 control</th>
<th>1 treatment</th>
<th>2 control</th>
<th>2 treatment</th>
<th>3 control</th>
<th>3 treatment</th>
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<td>10^4</td>
<td>10^5</td>
<td>10^3</td>
<td>10^4</td>
<td>10^5</td>
</tr>
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</table>

Biofilms from three patients with large (>10 cm^2) venous ulcer were split into two tubes containing saline (control) or saline with 200 ug/ml gentamicin (treatment), and after 24 hours of incubation, samples were dispersed into microcolonies and CFU/gm were measured. Total counts of bacteria at 0, 1, and 3 days after initial debridement remained consistently high. However, in two of the wounds, all bacterial were "planktonic" at 1 and 2 days after debridement (full kill by exposure to gentamicin), but by 3 days post-debridement, all three wounds had re-established substantial levels of biofilm bacteria (10^3–10^5 CFU/gm).


**Question:** Does NPWT with instillation (NPWTi) of microbicidal solutions effectively kill mature biofilms grown on pig skin explants?

**Answers:**

1. No killing of biofilm CFUs
2. Total killing of biofilm CFUs
3. Varying levels of killing biofilm CFUs depending on the composition of the instillation solution

**Assessment of VeraFlow® Prototype Therapies on Mature Biofilms Grown on Pig Skin Explants**

1. Create a deep partial thickness injury (0.8 mm deep) measuring 2 inches wide by 4 inches long (50 mm x 100 mm) to explant of pig skin (18 cm X 14 cm)
2. Sterile with bleach and chlorine gas
3. Explants are placed onto a 500 cm² dish containing 0.5% soft tryptic soy agar with antibiotic and antifungal
4. Inoculated with solution containing 10^5 cfu of P. aeruginosa
5. Grow for 4 days at 37°C with daily changes to fresh soft agar plates with antibiotic (gentamicin).
PLACEMENT OF TWO PORT VAC-INSTILL DRESSING
A KCI open cell foam pad is placed over the wound surface, and a two port V.A.C. Instill® dressing is placed over the pad and connected to the V.A.C. Instill® Unit.

METHODS: Four assembled test units with pig skin explants in large 37°C incubator

3.0 METHODS: Multiple Biopsies Taken
Explants are cultured for 24 hours at 37°C with 6 cycles of VeraFlow® treatment which consists of 4 hours of negative pressure followed by 30 seconds of Instill (delivers 50 ml of solution) then 10 minutes of dwell per cycle.

Multiple 8 mm punch biopsies were taken, placed in 5 ml of culture broth, sonicated for 5 cycles of 90 seconds with 1 minute delay between cycles then vortexed, spread on culture plates and colonies are counted 24 hours later. Central biopsies are processed for SEM.

Effects of 6-Cycles of NPWT-Instill Treatments Over 24 Hours on *P. aeruginosa* Biofilm Grown on Pig Skin Explants

Effects of 24 Hours of VerFlow® Prototype Treatments (6-Cycles) on *P. aeruginosa* Grown on Pig Skin Explants

SEM After 6-Cycles of NPWTi Over 24 Hours

 Effects of 6-Cycles of NPWT-Instill Treatments Over 24 Hours on *P. aeruginosa* Biofilm Grown on Pig Skin Explants

*P-Value <0.005 compared to saline control

**SEM After 6-Cycles of NPWTi Over 24 Hours**

Plains, Q. Yang, G. Schultz, International Wound J, in press
**Larval Debridement Therapy**

**Effect Of Ultrasonic Cleansing On Mature Biofilms On Pig Skin Explants**

- Contact or noncontact ultrasonic cleansing alone has minimal effects on mature biofilms (*P. aeruginosa*) (Schultz unpublished data)
- Combining ultrasonic cleansing with selected microbicidal fluids significantly reduces levels of bacteria in mature biofilms (Schultz, unpublished data)

**Summary**

1. Biofilms are communities of bacteria encased in a self-produced matrix of polysaccharides, protein and DNA that provides high levels of tolerance to antibodies, antibiotics and antiseptics
2. Biofilms are present in a high percentage of chronic wounds and they impair healing by stimulating chronic inflammation, leading to elevated levels of proteases and ROS that degrade proteins that are essential for healing
3. Topical dressings can reduce biofilm CFUs ~1 to 2 logs except sustained release cadexomer iodine dressings - totally kills biofilm
4. NPWT alone has minimal effects on reducing mature biofilms when tested using an in vitro pig skin explant model
5. NPWT + Instillation of some wound cleansing solutions significantly reduced CFUs of *P. aeruginosa* biofilms compared to NPWT control
6. Larval debridement and MIST cleansing with microbicidal solutions reduce biofilms in vitro
7. Clinical studies are needed to assess effects in patients

**Extra Background Slides**

**Effects of Non-Contact Ultrasonic Wound Cleansing on Biofilms**

**Different Effects of Silver Containing Dressings on Biofilms**


**TABLE 1. Characteristics of biofilms exposed to silver dressings for 1 and 7 days in a daily transfer assay**

<table>
<thead>
<tr>
<th>Organism and parameters</th>
<th>1st day</th>
<th>7th day</th>
<th>MIN</th>
<th>MAX</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em> lipids</td>
<td>15.5±3.1</td>
<td>8.5±2.1</td>
<td>2.0±0.4</td>
<td>6.5±1.9</td>
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<tr>
<td><em>P. aeruginosa</em> saccharides</td>
<td>15.5±3.1</td>
<td>8.5±2.1</td>
<td>2.0±0.4</td>
<td>6.5±1.9</td>
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<tr>
<td><em>P. aeruginosa</em> proteins</td>
<td>15.5±3.1</td>
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<td>2.0±0.4</td>
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<td><em>P. aeruginosa</em> nucleic acids</td>
<td>15.5±3.1</td>
<td>8.5±2.1</td>
<td>2.0±0.4</td>
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