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A mucosal model to study microbial biofilm development and anti-biofilm therapeutics

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Abstract

Biofilms are a sessile colony of bacteria which adhere to and persist on surfaces. The ability of bacteria to form biofilms is considered a virulence factor, and in fact is central to the pathogenesis of some organisms. Biofilms are inherently resistant to chemotherapy and host immune responses. Clinically, biofilms are considered a primary cause of a majority of infections, such as otitis media, pneumonia in cystic fibrosis patients and endocarditis. However, the vast majority of the data on biofilm formation comes from traditional microtiter-based or flow displacement assays with no consideration given to host factors. These assays, which have been a valuable tool in high-throughput screening for biofilm-related factors, do not mimic a host-pathogen interaction and may contribute to an inappropriate estimation of the role of some factors in clinical biofilm formation. We describe the development of a novel *ex vivo* model of biofilm formation on a mucosal surface by an important mucosal pathogen, methicillin resistant *S. aureus* (MRSA). This model is being used for the identification of microbial virulence factors important in mucosal biofilm formation and novel anti-biofilm therapies.

1.0 Introduction

Biofilms are communities of micro-organisms that are embedded in an extracellular matrix composed of proteins, lipids, polysaccharides and nucleic acids. The members of a biofilm are protected from environmental factors such as UV light and dehydration, as well as from host immune cells such as neutrophils and other phagocytes (Hall-Stoodley et al., 2004). Biofilm associated bacteria are also much more resistant to antimicrobial agents (Stewart and Costerton, 2001).

Recently there has been a great deal of interest in the role of biofilms in infectious diseases. The National Institutes of Health has estimated that ~80% of human infections are caused by pathogenic biofilms, (SBIR/STTR study and control of microbial biofilms, <http://grants.nih.gov/grants/guide/pa-files/PA-99-084.html>). The sites of biofilm-mediated infections include temporary or permanent medical devices (endotracheal tubes,

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intravascular and urinary catheters, orthopedic implants and arterial stents) and wounds, blood, and mucosal surfaces (sinuses, respiratory and genito-urinary tracts) (Bakaletz, 2007, Donlan, 2001, Wolcott et al., 2008).

The mucosa represents a surface where microbes from the external environment interact with host tissues (Kaufmann et al., 2011). Often, these interactions are beneficial or benign, as in the case of commensal organisms. However, the mucosa remains a major site of entry for pathogens. Barrier function alone is often insufficient in protecting against microbial pathogens. Therefore, the mucosa also contains cells and soluble regulatory and effector molecules of the innate and adaptive immune systems (Janeway and Medzhitov, 2002). These constituents include epithelial cells, neutrophils, macrophages, dendritic cells, natural killer cells, T and B lymphocytes, mucin and a variety of effector peptides and proteins such as defensins, complement, C-reactive protein, and pro-inflammatory chemokines and cytokines (Kaufmann, Rouse and Sacks, 2011). These serve as critical components of the host immune response to infections.

The study of biofilm formation on biologically relevant surfaces, such as mucosal tissue, allows for a clearer understanding of host/pathogen interactions as the model system used can have profound effects on the expression of potential virulence factors involved in biofilm formation (Costerton et al., 1987, Otto, 2008). In fact, it has been demonstrated that the substrate used for biofilm formation can impact bacterial gene expression (Anderson et al., 2008, de Breij et al., 2009). These studies underscore the importance of using a biological matrix for studies of medical biofilm growth that will inform the development of treatments for clinical infections.

In vivo studies are the gold standard in pathogenesis, but they are expensive and labor intensive. Co-cultures of pathogens and mammalian cell lines, while contributing valuable information, can only be conducted over short periods of time (i.e. less than 24 hours). This is due to the cytotoxic effects of the pathogens on the cells, which reduces the utility of these studies as biofilms can take multiple days to reach maturity. For example, it was recently shown U2OS osteosarcoma cells infected with *S. aureus* or *Pseudomonas aeruginosa* rounded up and detached by 18 h post-infection, however, growth of both the U2OS cells and the less virulent, commensal organism *S. epidermidis* was observed as late as 48 h (Subbiahdoss et al., 2011). Reconstituted human epithelial tissue cultures have been used as a surrogate for *in vivo* *C. albicans* biofilm studies, but the construction of the 3-dimensional tissue culture is also labor intensive and expensive. Recognizing these limitations, we developed an *ex vivo* full thickness tissue model to study biofilm formation by clinical isolates of MRSA, an important pathogenic bacteria.

The human vaginal surface is comprised of non-keratinized, stratified, squamous epithelium and interspersed cells of the immune system covered by a layer of mucus (Thompson et al., 2001). *S. aureus* commonly colonizes mucosal surfaces such as the vagina and the anterior nares (Lowy, 1998). Colonization has been associated with an increased risk of *S. aureus* infections (Kluytmans et al., 1995). Up to 10% of women are colonized vaginally with *S. aureus* (Martin et al., 1982) and as many as 20 – 60% of people are colonized in the anterior nares persistently and transiently, respectively (Kluytmans et al., 1997).

We selected porcine vaginal mucosa (PVM) as our *S. aureus* biofilm model substrate for multiple reasons. Most infections initiate at mucosal surfaces and *S. aureus* colonizes the human vaginal mucosa. Like its human counterpart, PVM consists of stratified, squamous epithelium, protected by a layer of mucus (Kong and Bhargava, 2011, Squier et al., 2008). The porcine vagina is relatively large in size, so that many small biopsies may be obtained from a single specimen, which in turn allows us to test multiple variables in our experiments

without concerns of inter-animal variability. It is inexpensive and easy to procure. Finally, the culture of a stratified, squamous epithelium and underlying connective tissue allows us to study mature biofilm formation over the course of 3 days.

Here we present a novel mucosal biofilm model, which enables the study of biofilm formation and the factors that contribute to a biofilm phenotype by MRSA, in an environment mimicking a natural infection. This model can also be utilized as a semi-high throughput platform for novel anti-biofilm therapy development, as not only can we assess efficacy, but also host cytotoxicity. We used the MRSA mucosal biofilm model to demonstrate a lack of efficacy by 0.12% chlorhexidine gluconate (CHG, Peridex™) on biofilm formation. CHG is an antiseptic agent commonly used in oral rinses within the dental community and more recently for prevention of ventilator-associated pneumonia (VAP). CHG has also been used to decolonize the vagina prior to delivery for prevention of transmission of β -hemolytic *Streptococcus* as well as treatment of vaginal infections in non-pregnant women, at concentrations ranging from 0.25 to 2% (Goldenberg RL, 2006, Molteni et al., 2004, Wilson et al., 2004).

2.0 Methods

2.1 Tissue preparation and bacterial culture

Normal healthy porcine vaginal tissue was excised from animals at slaughter (Andrew Boss Laboratory of Meat Science, University of Minnesota, St. Paul, MN) and transported to the laboratory in RPMI 1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA), penicillin (50 IU/mL, MP Biomedicals, Solon, OH), streptomycin (50 mg/mL, MP Biomedicals, Solon, OH) and amphotericin B (2.5 μ g/mL, Hyclone, Logan, UT). Antibiotics were included to decolonize normal flora which may interfere with biofilm formation. Tissue was utilized within 3 hours of excision. Explants of uniform size were obtained from the porcine vagina using a 5mm biopsy punch. Excess muscle tissue was trimmed away with a scalpel. Tissue explants were washed in antibiotic-free RPMI 1640 media 3 times. The explants were then placed mucosal side up on a PET track-etched 0.4 μ m cell culture insert (BD Bioscience, Franklin Lakes, NJ) in 6-well plates containing fresh serum-free and antibiotic-free RPMI 1640 and incubated at 37°C. The mucosal surface was continually exposed to air.

A biofilm forming bioluminescent strain of MRSA (Xen30, parent strain is a clinical isolate from Roche) was purchased from Caliper Life Sciences (Caliper Life Sciences, Hopkinton, MA). This isolate was selected for its previously characterized capability to form robust biofilms *in vitro* and *in vivo*. Stationary phase (overnight) cultures of MRSA were washed in RPMI 1640 and resuspended to a concentration of approximately 5×10^8 CFU/mL. Two μ l of this suspension were then used to inoculate tissue explants on the mucosal surface (1×10^6 CFU/explants). Explants were returned to 37°C and incubated for 0 – 72 h.

2.2 MTT evaluation of tissue viability

Viability of the explants was quantified by the reduction of the tetrazolium salt 1–4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) by live tissue into an acidified isopropanol soluble product (CGDA kit, Sigma, St. Louis, MO). Briefly, after culturing for the indicated amount of time, explants were transferred to 96-well tissue culture plates containing 100 μ l serum-free, antibiotic-free RPMI 1640 and 10 μ l MTT substrate. The explants were returned to the 37°C incubator for 3 h. After incubation, the purple formazan product was extracted using acidified isopropyl alcohol at 4°C for 18 h. Optical density in the wells was measured following removal of explants. Data are expressed relative to fresh tissue which was used as the maximum viability control.

2.3 Enumeration of CFU

Bacteria were enumerated from infected explants by homogenization (highest setting for 30 s) or vortex mixing (highest setting, 2 m) in 250 μ l sterile phosphate buffered saline (PBS, Sigma, St. Louis, MO) or CHG neutralizing solution containing Triton X-100 (Aldrich, St. Louis, MO), lecithin (Alfa Aesar, Ward Hill, MA), Tween 80 (Sigma, St. Louis, MO) and sodium thiosulfate (Sigma, St. Louis, MO). Homogenates/supernatants were then serially diluted in PBS (or plated neat) and spread on Tryptic Soy Agar containing 5% sheep's blood (Beckton Dickinson, Franklin Lakes, NJ) using a spiral plater (Biotekw, Microbiology International, MD). In some experiments, explants were gently washed 3 $\times$ in PBS prior to vortex mixing to count planktonic vs. adherent (biofilm) bacteria.

2.4 Scanning electron microscopy

Infected and control explants were washed gently in normal saline (Beckton Dickinson, Franklin Lakes, NJ). They were then fixed and processed as previously described (Erlandsen et al., 2004). Briefly, explants were fixed in 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) + 2% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) + 0.15% alcian blue (Sigma-Aldrich, St. Louis, MO) in 0.15 M cacodylate buffer for 22 h. Specimens were washed in 0.15 M cacodylate (Electron Microscopy Sciences, Hatfield, PA) buffer and then post-fixed in 1% OsO₄ (Electron Microscopy Sciences, Hatfield, PA) + 1.5% potassium ferrocyanide (Sigma-Aldrich, St. Louis, MO) in 0.15 M cacodylate buffer for 90 m, protected from light. Specimens were again washed 3x in 0.15 M cacodylate buffer, then dehydrated in an ethanol series: 50%, 70%, 80%, 95% and 2x 100% for 5 m per step. After critical point drying in CO₂, specimens were mounted mucosal side up on SEM stubs using adhesive carbon tabs (Ted Pella, Redding, CA). Specimens were sputter coated with approximately 1–2 nm platinum and imaged on a Hitachi S-4700 field emission SEM at 2keV.

2.5 Laser scanning confocal microscopy

Infected and control explants were stained using FilmTracer™ LIVE DEAD® Biofilm Viability kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. After staining, specimens were gently washed 3 x in Hank's balanced salt solution and transferred to glass slides. A coverslip with 1 mm spacer (Electron Microscopy Sciences, Hatfield, PA) was then applied and specimens were imaged on an Olympus Fluoview 1000 BX2 (Olympus America Corporation, Center Valley, PA) using a 60 x oil immersion objective. Images were captured and processed using Fluoview software (Olympus America Corporation, Center Valley, PA).

2.6 Immunohistochemistry

Explants which had been infected for 72 h were rinsed once in HBSS (Invitrogen, Carlsbad, CA). A concentrated solution of purified human IgG (75 μ g, Sigma, St. Louis, MO) was then applied for 30 min to block non-specific antibody binding by *S. aureus* protein A. Next anti-PNAG goat serum (1:100 dilution in HBSS, a kind gift of G. Pier, Harvard Medical School, Boston, MA) or normal goat serum (1:100 dilution in HBSS, Sigma, St. Louis, MO) was applied and allowed to incubate at RT for 2 h. Following incubation, antibodies were removed by pipetting and explants were soaked in 1 mL fresh HBSS for 5 min. Donkey anti-goat Alexa Fluor® 488 (1:250 dilution in HBSS, Invitrogen, Carlsbad, CA) was then applied and explants were incubated for 1 h at RT, protected from light. Hoechst dye (1:5000 dilution in HBSS, Invitrogen, Carlsbad, CA) was then applied and incubated for 15 min at RT, protected from light. Explants soaked in fresh HBSS for 5 min, then were washed 2 more times in HBSS and then mounted on glass slides and cover-slipped using a 1 mm coverwell imaging chamber (Electron Microscopy Sciences, Hatfield, PA). Specimens

were imaged on an Olympus Fluoview 1000 BX2 (Olympus America Corporation, Center Valley, PA) using a 60x oil immersion objective. Images were captured (with a 3 x zoom) and processed using Fluoview software (Olympus America Corporation, Center Valley, PA).

2.7 Anti-biofilm efficacy of Peridex (0.12% CHG)

Infected explants were treated with 10 μ l of PeridexTM solution (3M Company, St. Paul, MN) either 2 h post-infection or 48 h post-infection. Explants were returned to 37°C incubator. Twenty-four hours post application of PeridexTM, explants were transferred to tubes containing 250 μ l of CHG neutralizing solution [a phosphate (Sigma, St. Louis, MO) buffer containing Triton X-100 (Aldrich, St. Louis, MO), lecithin (Alfa Aesar, Ward Hill, MA), Tween 80 (Sigma, St. Louis, MO) and sodium thiosulfate (Sigma, St. Louis, MO)] and vortex mixed on high for 2 min. Supernatants were plated neat or serially diluted in PBS and CFU were enumerated after overnight growth.

2.8 Statistical analysis

Each experiment was performed in triplicate and repeated a minimum of two times. Data presented are mean $\pm$ s.e.m of triplicates. Statistical analysis (Student's T test) was performed using GraphPad Prism Software (GraphPad Software, Inc., La Jolla, CA).

3.0 Results

3.1 Experimental design and MRSA growth on a mucosal surface

Normal specimens of porcine vaginal tissue were procured from The Andrew Boss Laboratory of Meat Science at the University of Minnesota and used within 3 hours (h). Uniform sized explants (5 mm diameter) were cut from the vaginal tissue using a biopsy punch (Fig. 1a, b). Explants were prepared in RPMI 1640 media supplemented with antibiotics to decolonize normal flora. Explants were then washed in unsupplemented media and transferred to 6-well tissue culture plates containing 1 mL unsupplemented RPMI media and a transwell insert with a membrane pore size of 0.4 μ m (Fig. 1c, d). The explants were placed mucosal side up on the transwell membrane. Using an MTT based assay, we demonstrate that the viability of uninfected tissue explants is unchanged for up to 6 days, Fig. 1e. An inoculum of $\sim 1 \times 10^6$ CFU MRSA (Xen30) was applied to the surface of the mucosa in 2 μ l. This volume was optimized for inoculation as it forms a bead on the surface of the mucosa. Additionally, the pore size of the insert membrane (0.4 microns) is too small for *S. aureus* to penetrate; therefore infection of the tissues was strictly from the apical (luminal) side of the mucosa.

3.2 Growth and adhesion of MRSA to PVM

After inoculation, explants were incubated at 37°C, 7% CO₂ for the indicated times. Logarithmic growth of MRSA was observed between 6 h to 24 h of culture, followed by a stationary phase which persisted through the remainder of the experiment (48 h, Fig. 2a). The proportion of bacteria adherent to the tissue increased over time (Fig. 2b). Twenty-four hours post infection, only approximately 7% of the bacteria are adherent when the tissue is gently washed [7.28 ± 0.25 (s.e.m.) log₁₀ CFU/explant out of a total of 8.06 ± 0.10 (s.e.m.) log₁₀ CFU/explant, Fig. 2b]. This proportion significantly increases over time, such that by 48 h, $\sim 63\%$ of the bacteria remain adherent [8.02 ± 0.06 (s.e.m.) log₁₀ CFU/explant vs. 8.21 ± 0.06 (s.e.m.) log₁₀ CFU/explant, adherent + planktonic, Fig. 2b]. This proportion drops slightly but remains constant at $\sim 50\%$ throughout the remainder of the experiment.

3.3 Visualization of MRSA infection of vaginal mucosa and subsequent biofilm formation by scanning electron microscopy

Scanning electron microscopy of the uninfected vaginal luminal surface at 24 h revealed a layer of flattened squamous epithelial cells with occasional partially detached cells (Fig. 3a). At 48 h post initiation of tissue culture, the epithelium remains largely intact, with occasional partially detached squamous epithelia observed (Fig. 3b). In contrast, to uninfected tissue, 24 h after infection with MRSA nearly all of the epithelial cells have become rounded, indicating detachment from adjacent epithelia (Fig. 3c). Attached Staphylococci were rare, however, a cluster of Staphylococci attached to the epithelium is depicted in Fig. 3e at higher magnification (10,000 x). Forty-eight hours post infection with MRSA, microcolonies of Staphylococci are observed among detached epithelia (Fig. 3d). A higher magnification (10,000 x) image reveals Staphylococci secreting extracellular matrix and embedded in matrix as well as the presence of some epithelial cells (Fig. 3f).

3.4 Visualization of MRSA mucosal biofilms by confocal laser scanning microscopy

We next employed the use of LIVE/DEAD® staining in conjunction with confocal laser scanning microscopy (CLSM) to simultaneously visualize living or dead epithelial cells and biofilm development. LIVE/DEAD® staining of the uninfected vaginal mucosa revealed live epithelia (green) and intact mucosal tissue throughout the course of experimentation, with occasional dead (red) cells (Fig. 4a–c). In contrast, 24 h post infection, MRSA has killed nearly all of the epithelia as indicated by the red staining (Fig. 4d). MRSA infection for 48 h resulted in further destruction of the mucosal epithelia (red, black) as well as the formation of staphylococcal microcolonies (green, Fig. 4e). Mature biofilm is formed by 72 h post infection and few dead epithelia remain (Fig. 4f).

3.5 Confirmation of biofilm formation by PNAG staining and confocal laser scanning microscopy

To confirm that the large masses of live cocci we observed via LIVE/DEAD® staining and CLSM of 72 h infected tissue are in fact embedded in a biofilm, we employed antigen-specific staining for poly-*N*-acetylglucosamine (PNAG), the main component of the *S. aureus* biofilm matrix. Staining with goat-anti-PNAG (a kind gift of G. Pier) followed by donkey anti-goat Alexa Fluor 488® and Hoechst 33342 dye revealed both diffuse and punctate green staining (Fig. 5a). Specificity was demonstrated by a lack of green staining with normal goat serum followed by the labeled donkey secondary antibody. Sparse blue epithelial cell nuclei are visible (Fig. 5b), consistent with the LIVE/DEAD® stain which demonstrated a few red (dead) remaining epithelial cells at this late time point.

3.6 Chlorhexidine gluconate (0.12%, Peridex™) is ineffective at killing mucosal MRSA biofilm

Chlorhexidine gluconate (CHG) is an antiseptic commonly used for infection prevention in the hospital setting and by the dental community. Biofilms are known to be much more resistant to antimicrobials than their planktonic counterparts, therefore we examined the effect of CHG on planktonic vs. biofilm MRSA on PVM. The concentration of CHG commonly used in the oropharyngeal cavity is 0.12% and we have demonstrated previously that this concentration is sufficient to kill *S. aureus* in their planktonic form (Anderson et al., 2010). After 24 h exposure, Peridex™ (0.12% CHG, active ingredient) was found to be non-toxic to the PVM (data not shown). Explants of PVM were prepared as described above and infected for either 2 h (planktonic) or 48 h (biofilm) prior to treatment with Peridex™ for 24 h (Fig. 5). We observed that approximately 1.5 log₁₀ MRSA were killed by the application of Peridex™ 2 h post infection (prior to biofilm formation) of PVM (Fig. 5a). This was significantly different ($p < 0.05$, Student's t-test, $n = 3$, $\text{mean} \pm \text{s.e.m.}$) from untreated, MRSA

infected tissue. In fact, MRSA on untreated tissue continued to grow ($2 \log_{10}$) above the initial inoculum of $6 \log_{10}$ CFU to $\sim 8 \log_{10}$ CFU/explant. In contrast, when CHG was applied to MRSA biofilm microcolonies for 24 h, similar amounts of bacteria were recovered from treated and untreated control ($\sim 8 \log_{10}$ CFU/explant, Fig. 5b).

4.0 Discussion

Current models to study microbial biofilm formation include *in vitro* biofilm systems, cell-culture-based systems and *in vivo* animal models (Coenye and Nelis, 2010). Our model has several advantages over the strictly plastic *in vitro* models such as microtiter plates, flow cells and CDC reactors. We are able to evaluate the host response and protein contribution to biofilm formation, which more closely mimics an *in vivo* situation. For example, in these studies, we observed a large amount of epithelial cell cytotoxicity 24 h post exposure to the organism. Additionally, we are able to identify bacterial virulence factors important for biofilm formation on mucosal tissues. We reported previously that epithelial cytotoxicity was differentially affected by different strains of Toxic Shock Syndrome (TSS) isolates of *S. aureus*, in spite of the strains being equally inflammatory to the PVM (Anderson et al., 2012). We further determined that menstrual TSS isolates which contained a pseudogene for the secreted cytolysin alpha-toxin did not form a biofilm on mucosal tissue, whereas a pneumonia TSS isolate which contained the wild type alpha-toxin gene formed a robust biofilm, similar to that observed in this study. Further, we and others have shown that host factors diminish the activity of some antiseptic agents, underscoring the importance of the model system when evaluating novel anti-biofilm agents (Anderson, Horn, Lin, Parks and Peterson, 2010, Gelinas and Goulet, 1983, Oliveira et al., 2010). Our model retains this translational benefit, whilst having the additional benefit of being semi-high throughput.

We demonstrate that the logarithmic growth phase is followed by a period during which the biofilm is established, reflected in the rise in percentage of organisms adherent at 72 h versus 24 h. Although scanning electron microscopy can be subject to over-interpretation of artifacts, the images indicate biofilm formation by the staphylococcal organisms. Further, the LIVE/DEAD[®] staining and immunohistochemistry combined with CLSM support the formation of a stable biofilm by 72 h, replacing the squamous mucosa which otherwise remained morphologically normal in the absence of organisms. While from a purely biological perspective the ability to form reproducible biofilm models is significant, the value of the model to translational research lies in its ability to reflect what is known about clinical biofilm behavior. CHG has been shown to be an effective agent against most organisms in the planktonic state but has been unsuccessful in eradicating biofilms clinically and our model recapitulates these findings.

Cell-culture models have been developed to mimic an *in vivo* state, but they are limited. For example, little similarity is observed in the biofilm *P. aeruginosa* response to sub-inhibitory concentrations of tobramycin in the presence of airway epithelial cells when compared to planktonic bacterial cells and biofilms grown on granite (Anderson, Moreau-Marquis, Stanton and O'Toole, 2008). The data presented in this paper suggest that *P. aeruginosa* biofilms behave differently depending on whether the substrate is organic in nature or abiotic. One drawback to the co-culture model is the limited amount of time the bacteria may be cultured with the cells due to cytotoxicity. The authors were fortunate, however, in that they observed biofilm formation in the limited (9 h) timeframe.

In vivo models have been developed to study biofilm formation on medical devices as well as pathogenic biofilm infections of mucosal surfaces. *In vivo* models not only allow for the study of pathogenesis of biofilms or the growth of the biofilm on a medical device, but have the added advantage of allowing for investigation of the microorganisms' dissemination to

other anatomical locations. A rat central venous catheter model was developed to study staphylococci biofilm formation as these types of infections cause significant morbidity and mortality in the hospital setting (Ulphani and Rupp, 1999). Other *in vivo* models include subcutaneous foreign body, intraperitoneal foreign body infection, urinary tract infection, otitis media, respiratory infection, and osteomyelitis models (Carsenti-Etesse et al., 1992, Christensen et al., 1983, Christensen et al., 2007, Fluckiger et al., 2005, Gerhart et al., 1993, Giebink et al., 1976, Kristian et al., 2003). These models have been used to study the efficacy of antibiotics, the effects of antimicrobial-impregnated implants, and the genetic determinants of pathogenesis. They all represent expensive, labor intensive, time-consuming experiments subject to regulation due to the use of vertebrate animals.

Our model has several benefits over the use of whole animals (Squier, Mantz, Schlievert and Davis, 2008). For example, the use of many small explants from one vaginal specimen provides convenience, efficiency, and semi-high throughput experimentation. Further, samples of porcine tissue are inexpensive to obtain and handling is simple when compared to the use of whole animals or human tissue. Another important consideration is that porcine tissue is not subject to regulation as it is a by-product of the slaughter of animals for food. Also, the PVM has been validated as similar to human vaginal mucosa. The PVM model does have limitations compared to the use of whole animals. For example, there is no blood supply, which would allow for influx of immune cells to the site of infection. Additionally there is no mechanism for the study of biofilm dispersal as the model is static in nature.

CHG is a powerful antiseptic which has been used in the reduction of dental plaque. More recently, it has been suggested that CHG may be of use in decontamination of the oral cavity of mechanically ventilated patients to reduce the incidence of ventilator associated pneumonia (VAP) (Labeau et al., 2011). VAP is a common complication of mechanical ventilation occurring in as high as 28% of ventilated patients, 24 – 50% of which result in mortality (Chastre and Fagon, 2002). The hypothesis is that a reduction in oral microorganisms will result in a reduction in VAP, which develops after aspiration of oropharyngeal secretions containing oral bacteria. Studies aimed at proving this have yielded mixed results. For example, DeRiso et al. observed a 69% reduction in nosocomial respiratory infection rates when 0.12% CHG oral rinse was used once preoperatively and twice per day postoperatively (DeRiso et al., 1996). In contrast, Bellissimo-Rodrigues et al. observed no difference in the rate of respiratory infections in ventilated patients who received 0.12% CHG oral rinse 3 times per day compared to the placebo control group, although the authors noted that CHG may slow the onset of infection (Bellissimo-Rodrigues et al., 2009). More recently, in a randomized, double blind, placebo controlled clinical trial of pediatric patients, application of 0.12% CHG twice per day failed to significantly impact the incidence of VAP (Kusahara et al., 2012). Our data demonstrate that Peridex™ does not disrupt MRSA biofilm and may explain the failure of CHG in clinical trials. Mechanistically, Peridex's™ failure to kill MRSA biofilm could be that CHG could not penetrate the biofilm matrix and reach the bacterial membranes, which is where CHG has its activity. Alternatively, it could be that CHG was sequestered by substrate (host) proteins or membranes, effectively neutralizing it.

By understanding the microbial pathogenesis of biofilm formation in a system which more closely mimics natural biofilm infections, strategies to treat and prevent biofilm-mediated infection can be developed and utilized. Further, screening of novel, potential biofilm treatments or preventatives in a model which incorporates the host contribution is more predictive of clinical efficacy and should streamline the process to market.

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Highlights

- Pathogenic biofilms comprise a majority of human infections.
- Most *in vitro* models used for biofilm study ignore the host contribution to their formation.
- We developed an *ex vivo* mucosal model with *Staphylococcus aureus*.
- We show that an antiseptic commonly used in the clinic is ineffective against *S. aureus* mucosal biofilms.
- We believe our model is more predictive of antiseptic clinical efficacy and pathogenesis.

Figure 1.

Experimental setup and viability of PVM. a. Approximately 1/2 of a vaginal specimen collected from one pig. b. 5 mm diameter explants are made using a biopsy punch and excess muscle and connective tissue is trimmed away with a scalpel. c. Cartoon depiction of the explants resting on the transwell membrane inside a well of a cell culture plate. This cartoon was adapted from a drawing depicted in Zaga-Clavellina *et al.* 2007. d. The explants are placed mucosa side up on the membrane of a transwell seated above 1 mL unsupplemented RPMI 1640 media in a 6-well tissue culture plate. e. Mucosal tissue remains highly viable over the course of the experiment.

Figure 2.

Growth and adhesion of MRSA on PVM increases over time. a. MRSA exhibits typical log phase growth from time 0 to 18 h post infection, followed by stationary phase growth through 72 h. b. One day post-infection, ~93% of the bacteria are planktonic (washed away). This proportion significantly decreases over time, such that by 2 d, only ~37% of the bacteria are easily washed off (n=6, p<0.05). The proportion which remain attached post-washing remains relatively constant throughout the remainder of the experiment at ~50%. These experiments were performed with 3 – 6 replicate explants. Each experiment was repeated 2 times, for a total N = 3. Error bars represent SEM.

Figure 3.

Scanning electron micrographs of PVM with and without MRSA infection. a. and b. Normal healthy vaginal mucosa at 24 and 48 h, 1000 x original magnification. c. and d. MRSA infected PVM, 1000 x original magnification. The epithelium is disrupted. Epithelia have lost cell junctions and become rounded. EC, epithelial cell. *S. aureus* is visible as small dark spots. As time increased, more *S. aureus* and less intact epithelium are observed. e. and f. MRSA infected PVM, 10,000 x original magnification. e. Individual, smooth, adherent staphylococci are apparent at 24 h post infection. f. As time progresses, the staphylococci become coated in an extracellular matrix. B, biofilm

Figure 4.

Time course of biofilm development is observed by LIVE/DEAD[®] staining and confocal laser microscopy. The use of Molecular Probes' FilmTracer[™] LIVE/DEAD[®] Stain allows us to image both the mucosal epithelium and biofilm associated bacteria. a–c. control (uninfected) porcine vaginal epithelia remain live (green) and intact (asymmetrical) throughout the course of the experiment. d. 24 h post infection, MRSA has killed a majority of the epithelial cells (rounded, red staining). Black areas depict exposed extracellular matrix. e. 48 h post infection, MRSA are visualized as large micro-colonies of biofilm (green) on dead epithelia (red). f. 72 h post infection, mature MRSA biofilm (green) has nearly covered the tissue. EC, epithelial cell, MC, microcolonies, B, biofilm, scale bars are 20 microns

Figure 5.

Confirmation of biofilm formation by PNAG staining and confocal laser scanning microscopy. A combination of anti-poly-*N*-acetylglucosamine (PNAG) antibodies and Hoechst 33342 dye allowed us to specifically image both the main component of the biofilm matrix and remaining epithelial cell nuclei 72 h post-infection with MRSA. a. Staining with anti-PNAG followed by donkey anti-goat conjugated to Alexa Fluor 488[®] and Hoechst 33342 reveals the presence of the biofilm exopolysaccharides (diffuse and punctate green) among sparse epithelial nuclei (blue). b. Infected vaginal mucosa stained with normal goat serum (negative control) followed by donkey anti-goat conjugated to Alexa Fluor 488[®] and Hoechst 33342 reveals a lack of the exopolysaccharide (no green) and sparse epithelial nuclei (blue). Scale bars are 20 microns.

Figure 6.

Peridex™ (0.12% CHG, active ingredient) is ineffective at killing biofilm form MRSA. a. Approximately 1.5 log₁₀ bacteria were killed by the application of Peridex™ 2 h post infection of PVM for 24 h. b. No difference is observed in the number of bacteria recovered between CHG-treated and untreated explants which were infected for 48 h prior to the application of Peridex™ for 24 h. These experiments were performed in triplicate. The experiment was repeated 2 times, for a total N = 3. Error bars represent SEM.