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Multispecies biofilm in an artificial wound bed—A novel model for *in vitro* assessment of solid antimicrobial dressings

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ABSTRACT Q2

Wound infections represent a major problem, particularly in patients with chronic wounds. Bacteria in the wound exist mainly in the form of biofilms and are thus resistant to most antibiotics and antimicrobials. A simple and cost-effective *in vitro* model of chronic wound biofilms applied for testing treatments and solid devices, especially wound dressings, is presented in this work. The method is based on the well-established Lubbock chronic wound biofilm transferred onto an artificial agar wound bed. The biofilm formed by four bacterial species (*Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis* and *Pseudomonas aeruginosa*) was stable for up to 48 h post-transplant. The applicability of the model was evaluated by testing two common iodine wound treatments. These observations indicate that this method enables assessing the effects of treatments on established resilient wound biofilms and is clinically highly relevant.

1. Introduction

Microbial biofilms are structured communities of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface (Costerton et al., 1999). Sessile and planktonic microbial cells are phenotypically and physiologically different (Donlan and Costerton, 2002). Bacteria forming biofilms are highly resistant to many traditional therapies. Bacteria in biofilms can adapt to a sessile state by down-regulating cellular activity and encapsulating in a massive structure of extracellular polysaccharides (Brady et al., 2008; Sutherland, 2001). There is a growing recognition that biofilms are one of the principal causes of wound chronicity (Wolcott et al., 2010). Over 90% of chronic wounds contain bacteria and fungi from the skin, oral mucosa, enteric tract or the environment. Together these bacteria form a multispecies biofilm construct (Attinger and Wolcott, 2012; Q3 Price et al., 2009). Novel treatments for wound biofilms have been recently developed, potentially saving many lives by preventing systemic infections (Wolcott et al., 2010).

In order to develop antimicrobial therapies and test treatments, it is essential to have appropriate microbiological models. Most methodologies used to study antimicrobials and test medical devices use planktonic microbial cultures (Costerton et al., 1999). Several wound biofilm models were described previously to study different aspects of wound biofilms. These models use multiple species and aim to mimic the polymicrobial nature of wound biofilms (reviewed by Coenye and Nelis, 2010). Werthén et al. (2010) developed a model of wound biofilm without a solid surface and grown in the presence of a simulated body fluid composed of peptone and foetal calf serum. One of the more sophisticated biofilm models is based on tissue-engineered skin (Charles et al., 2009).

The first chronic wound biofilm model was developed by Sun et al. (2008) at the Medical Biofilm Research Institute in Lubbock, Texas, and was named the "Lubbock chronic wound biofilm (LCWB) model". This model was shown to be a realistic *in vitro* multispecies biofilm which grows and matures rapidly, is cost effective and easy to set up. Only liquid or semi-solid substances with putative inhibitory effects on biofilm formation were tested on the LCWB model (Dowd et al., 2009). This model was also modified for the high throughput testing of anti-biofilm properties of different wound care products on staphylococcal biofilms (Brackman et al., 2013). Furthermore, the LCWB model was successfully transplanted into murine skin wounds to induce formation of wound biofilm (Dalton et al., 2011).

To our knowledge, few models were described for testing anti-biofilm activity of wound dressings and other solid materials. Lipp et al. (2010) used a drip-flow reactor model with monospecies biofilms only. Hammond et al. (2011) developed a burn wound "biofilm" model that comprised burn wound bacterial isolates grown on cellulose discs

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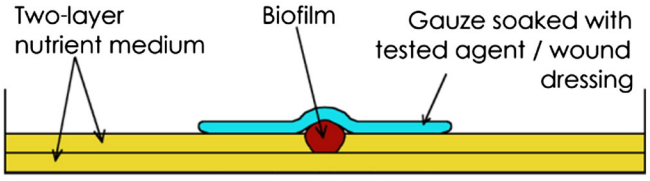

Fig. 1. Treatment of pre-formed biofilms transferred to artificial wound bed. Schematic drawing displays the cross-section of the Petri dish with a two-layer nutrient medium and centrally cultured biofilm covered with test antimicrobial agent/wound dressing.

and placed on agar plates. Different antibiotic ointments soaked in gauze were applied on the discs.

Recognizing the need for adequate *in vitro* biofilm models for evaluating solid anti-microbial wound dressings, we employed the well-established superior multispecies LCWB model. We transferred the pre-cultured biofilm onto an artificial wound bed and verified the applicability of this model for the testing of wound dressings. Here we describe the evaluation of this biofilm.

2. Materials and methods

2.1. Bacteria

Staphylococcus aureus, *Enterococcus faecalis*, *Bacillus subtilis* and *Pseudomonas aeruginosa*, originally isolated from patients with chronic infected wounds hospitalized at the University Hospital, Hradec Kralove (Czech Republic), were used in this study. Cryopreserved bacterial strains were grown for 24 h at 37 °C on Columbia agar plates supplemented with sheep blood (Oxoid, Germany). Sodium chloride peptone broth (buffered peptone bouillon, BPB; Merck, Germany) was generally used for dilutions and measuring optical density of cultures.

2.2. Modified Lubbock chronic wound biofilm (LCWB) model

We used the previously described LCWB model (Sun et al., 2008) with some modifications and amendments to pre-form the matured biofilms for treatment: briefly, 6 ml of liquid biofilm formation medium containing Bolton broth base (Sigma, Germany), 1% gelatine, 50% porcine plasma and 5% freeze-thawed porcine erythrocytes was dispensed into sterile 1.6 × 10 cm polystyrene tubes (Gama, Czech Republic). To control for possible variability in biofilm formation caused by the different batches of blood in the culture medium, four biological replicates were prepared in duplicate, each with blood from a different pig. Optical density-normalized cultures of four bacterial species were mixed together and 10 μl of 10^6 CFU/ml culture were inoculated into the tubes by ejecting the pipette tips along with the mixed bacterial suspension. The inoculated tubes were incubated at 37 °C in an orbital shaker (1.5 ×*g*) for up to 48 h. The biofilms were harvested at selected time intervals (12, 24, 36 and 48 h post-inoculation (p.i.)). Biofilms harvested 48 h post-inoculation were used to model chronic would biofilms and treated.

2.3. Treatment of established biofilms

Petri dishes with a two-layer nutrient medium composed of Bolton broth supplemented with 1% (*w*/*v*) gelatin and 1.2% (*w*/*v*) agar (Sigma, Germany) were prepared as follows: a 2-mm thin layer of nutrient medium was poured into Petri dishes. One sterile 20 × 8 mm PTFE-coated magnetic stirring bar was put onto the agar in each dish after solidification of the nutrient medium. A second 2-mm layer of nutrient medium was subsequently added. After the medium had completely congealed, the stirring bars were carefully and aseptically removed from the agar creating oval-shaped artificial wound beds. Pre-formed 48 hour-old biofilm was removed from the tube, washed with BPB and the pipette tip extracted from the biofilm using sterile forceps and a scalpel. The biofilm was placed into the "wound bed" in the nutrient medium and covered with a piece (2 × 5 cm) of 100% cotton 8-ply gauze sponge (Batist, Czech Republic), soaked with a test substance (Fig. 1). Biofilm cultures were incubated at 37 °C for 24 and 48 h respectively. After treatment, the biofilms were harvested from the artificial wound bed using sterile forceps and a Lang eye spoon, homogenized, and the bacteria were enumerated.

In our study aimed at model optimization and characterization, two commonly used antimicrobial wound treatments were applied to the biofilm model: polyvinypyrrolidone–iodine complex (0.2 mg of iodine/cm^2)–2 ml of 10% Alfadin (Bioveta, Czech Republic) per gauze and cadexomer-iodine complex (0.2 mg of iodine/cm^2)–2 ml of 11% Iodosorb gel (Smith and Nephew, USA), or 2 ml of concentrated Iodosorb gel per gauze (1.8 mg of iodine/cm^2), respectively. Gauze pieces soaked with 2 ml of BPB were used as controls.

2.4. Biofilm processing

Harvested biofilms were washed in BPB as follows; excess medium was removed with sterile cotton and biofilms were weighed. Subsequently, the biofilms were homogenized using a rotor-stator laboratory homogenizer (UltraTurrax, IKA, Germany). The biofilm homogenates were divided in three equal portions and used for quantification of biofilm bacteria and RNA isolation. Homogenates for molecular assays were resuspended in RNAlater (Life Technologies, USA), incubated overnight at 4 °C, pelleted and stored at −80 °C.

2.5. Quantitative cultures of biofilm bacteria

Homogenized biofilms were initially diluted 1:10 in BPB and vigorously vortexed for 2–3 min. The suspended cells were then diluted 10-fold, and 10 μl aliquots of each dilution and undiluted homogenate

Table 1
qPCR primers specifications.

Primer pair	Target	Sequences 5′–3′	Final concentration	Reference
16S	16S rDNA/rRNA	TCCTACGGGAGGCAGCAGT GGACTACCAGGGTATCTAATCCTGTT	100 nM	Nadkarni et al., 2002
SA	*S. aureus* *nuc*	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	300 nM	Hein et al., 2001
EF	*E. faecalis* 16S rDNA/rRNA	CCCTTATTGTTAGTTGCCATCATT ACTCGTTGTACTTCCCATTGT	500 nM	Rinttilä et al., 2004
PA	*P. aeruginosa* 16S rDNA/rRNA	CAAAACTACTGAGCTAGAGTACG TAAGATCTCAAGGATCCCAACGGCT	600 nM	Matsuda et al., 2007
BS	*B. subtilis* 16S rDNA/rRNA	CCCTTATTGTTAGTTGCCATCATT GGACTACCAGGGTATCTAATCCTGTT	100 nM	This study Nadkarni et al., 2002
icaA	*S. aureus* adhesin	TGAACCGCTTGCCATGTG CACGCGTTGCTTCCAAAGA	200 nM	Rode et al., 2007
ebrA	*E. faecalis* GntR family protein	TCGTCGTCATGGCAAAGGAA AGCAATCCGCAACCGACTTA	500 nM	This study