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Original Article

An in vitro Microfluidic Gradient Generator Platform for Antimicrobial Testing

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Abstract Methicillin Resistant *Staphylococcus pseudintermedius* (MRSP) biofilm-related infections are currently a leading concern for veterinary hospitals, as these types of infections are highly resistant to assaults by both the immune system and antimicrobial therapies, impeding their clearance. Research suggests that fosfomycin, a low molecular weight bactericidal antibiotic, has the potential to effectively penetrate and subsequently disrupt/destroy biofilm layers. Our study utilized a fabricated microfluidic gradient generator platform as an assay to perform a quantitative assessment of varying concentrations of a selected antimicrobial agent against MRSP biofilm formed under physiologically relevant conditions. Our results verified the feasibility of using a microfluidic device for rapid antimicrobial testing against biofilms, which was successful in demonstrating that fosfomycin is an effective agent that can disrupt established MRSP biofilms. Additionally, Atomic Force Microscopy (AFM) analysis revealed that the cell walls of MRSP cells within the biofilms were disrupted by fosfomycin treatment, which speaks to the mechanism of action and the antimicrobial efficacy of this agent. This study provides compelling evident that microfluidic device and nanoscale AFM imaging-based investigations of biofilms can aid in the study of biofilm-related infectious diseases.

Keywords: Microfluidics, *Staphylococcus pseudintermedius*, Fosfomycin, Biofilms, Microenvironment, AFM

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Introduction

One of the most prevalent adverse postoperative conditions accompanying veterinary surgery is a surgical site infection (SSI)[1]. SSIs represent a prominent portion of nosocomial infections that can lead to further complications; roughly 18% of the operations performed in cattle and companion animals results in SSIs[2-4]. In addition to the increased length of stay at the hospital after surgery, treatment and monitoring of SSIs result in a substantial economic burden on veterinary hospitals. An essential factor, which helps to both initiate and propel the infection process, is the ability of organisms associated with SSIs to form biofilms. Biofilms can protect constituent bacteria from shear and other inimical forces around the SSI by facilitating strong adhesion to biological surfaces, while simultaneously enhancing antimicrobial resistance[5,6,23]. As the adhesion of biofilm on surfaces increases, the elimination of biofilms by antimicrobials becomes more challenging due to the physiological changes that occur within these structures that ultimately enhance their resistance.

The widespread use of antibiotics over the last 50 years has helped to enhance the inherent resistance of several obligate and opportunistic pathogens, such as methicillin-resistant *Staphylococcus pseudintermedius* (MRSP), an opportunistic pathogen that is highly resistance to immune and antimicrobial assaults[7]. MRSP is considered to be the leading cause of hospital-acquired infections in companion animals, specifically canines[8-11]. Currently, no effective methods exist that can efficiently and completely eradicate MRSP-related biofilm infections. However, there are a few studies that suggest that fosfomycin, a broad spectrum antibiotic, may effectively penetrate and act upon SSI biofilms to effectively kill/eliminate both Gram-positive and Gram-nega-

tive bacteria (e.g. MRSP)[12-14].

One advantage offered by microfluidic platforms is the ability to precisely control the environment in which the sample under investigation is maintained. Microfluidics platforms can be used to analyze and subsequently characterize certain features of biofilm-forming microorganisms and non-biofilm formers alike by creating physiologically relevant microenvironments that are representative of the fluid composition and dynamics that might be found *in vivo*[15,21]. The primary virulence factor attributed to MRSP is its ability to form biofilms that are highly recalcitrant to removal/clearance[22]. Hence, the analysis of MRSP biofilm formation using such microfluidic platforms could provide insights into biofilm formation and its structure and potential remediation therapies. Here we evaluate the in vitro activity of fosfomycin against established MRSP biofilms within a microfluidic gradient-forming device, which we use to determine the minimum biofilm eradication concentration (MBEC). We subsequently contend that our microfluidic device is facile and can be used to conduct rapid drug toxicity assays, which could be used by other groups as our chamber can be readily fabricated, and although simple, it is highly effective compared to conventional microbiological assays.

Results and Discussion

Generation of the antimicrobial gradient

To visualize and correlate the concentration of fosfomycin that is applied to the biofilm across the observation module, we characterized the concentration gradient formed by the mixing module. Fluorescein solutions (50 mM, Sigma Aldrich, St. Louis, MO) in double-deionized water and phosphate buffered saline (PBS) were added to the device at a flow rate of 10 μL/h and imaged by an inverted epi-fluorescence microscope (LumaScope 500; Etaluma Inc., Carlsbad, CA). The microscope allowed for imaging at a single excitation/emission of 488 nm, which matches that of the fluorescence of the staining agent. The acquired fluorescent intensity profiles across the length of the observation module can be used to delineate any potential differences caused by diffusive mixing. The stability of the linear gradient was assessed by acquiring images at the connection point (0 mm) of the mixing module and then at increasing distances of 3, 6, and 9 mm from the connection point. We determined that the microfluidic platform was able to generate steady gradients along the observation module. The microfluidic device characteristic linear intensity was normalized to represent 16 μg/mL of fosfomycin (Figure 1a and 1b).

Quantification of MBEC against fosfomycin

To quantify the MBEC of MRSP biofilms, a suspended MRSP A12 strain was stained with Syto 9 (Molecular Probes, Invitrogen Corp, Carlsbad, CA) and then introduced inside the microfluidic device, thus allowing for the analysis of the antibacterial efficacy and the effect of dispersion of fosfomycin on the MRSP (Figure 2). The respective MBEC is determined by the borderline between the normalized fluorescence intensity, which is emitted by the biofilm and that of the

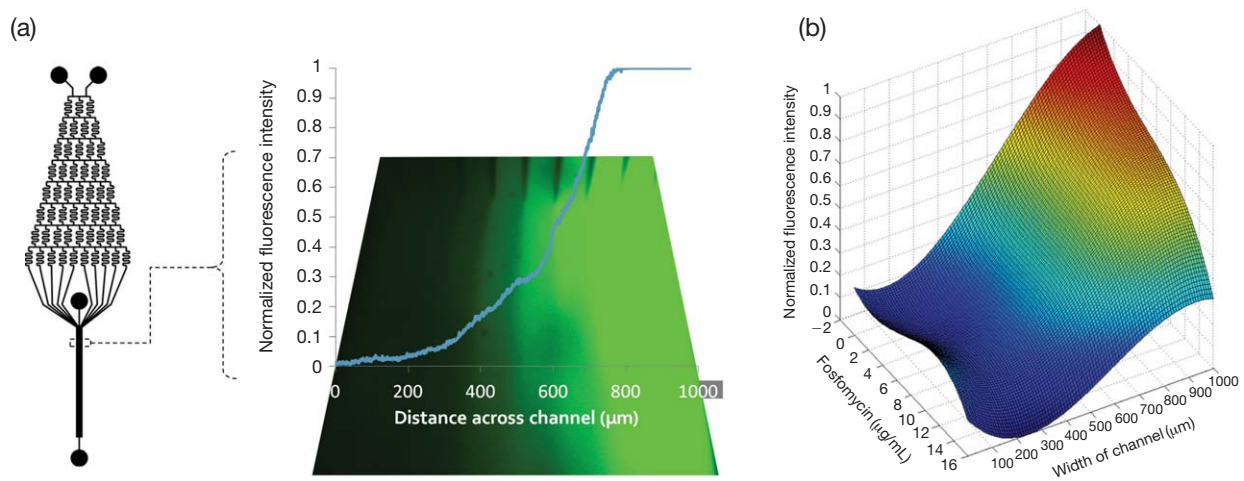

Figure 1. (a) Linear characterization of the microfluidic device-Fluorescein gradient across the channel width. (b). Relationship of normalized fluorescence intensity and fosfomycin (FOS) concentration (μg/mL) across the channel. The device's characteristic linear intensity was normalized to represent 16 μg/mL fosfomycin.

background across the width of the channel of processed images. Hence, the value of MBEC can be interpolated by plotting the normalized fluorescence intensity against the potential antimicrobial concentration across the width of the channel.

Three different concentrations of fosfomycin were used (16, 32, and 64 μg/mL) to investigate the remediation of the biofilm. The subsequent interpolations defined the MBEC value as 8.6±2.1 μg/mL of fosfomycin. In the microfluidic observation main channel (Figure 2), thicker biofilms were observed along the zone of low fosfomycin concentration. However, once the fosfomycin concentration began to increase, the respective cells started to detach and be washed away. To further validate our findings, we evaluated the reproducibility of the reduction in surface coverage of MRSP biofilms using a single concentration of fosfomycin (16 μg/mL). MIC serves as a standard assay for testing the antibiotic susceptibility of bacteria by testing bacteria's sensitivity in planktonic phase and is of limited value in determining the true antibiotic susceptibility, while MBEC provides direct determination of the bacteria in the biofilm phase of development[30].

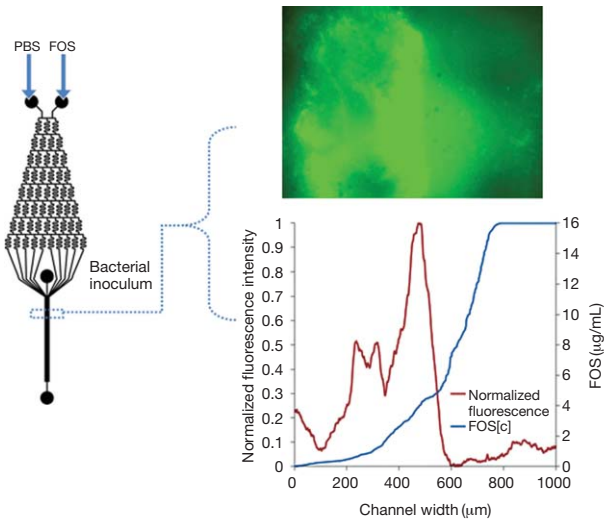

Figure 2. Microfluidic assay completed with 16 μg/mL fosfomycin applied after a 24 h growth period.

Figure 3 depicts the corresponding changes in surface coverage across three devices. The respective value of MBEC was interpolated as 8.1±0.9 μg/mL, thus indicating lower variation than tests comparing a range of inlet concentrations (8.6±2.1 μg/mL). It needs to be mentioned that our acquired results were in total agreement with a study that determined the minimum inhibition concentration (MIC) of a high biofilm-forming strain, MRSP A12, through a crystal violet microtitre plate assay (MPA); this helps to validate the results of the current study[16]. The former study provides clear evidence that a fosfomycin concentration of 0.8 μg/mL effectively reduces the amount of biofilm produced in an MPA by MRSP A12 to levels that are less than the corresponding negative control. The obtained results presented here support the efficacy of this microfluidic assay in the analysis of antimicrobial susceptibility of biofilm-embedded cells and the subsequent rapid assessment of multiple concentrations of therapy on MRSP biofilms.

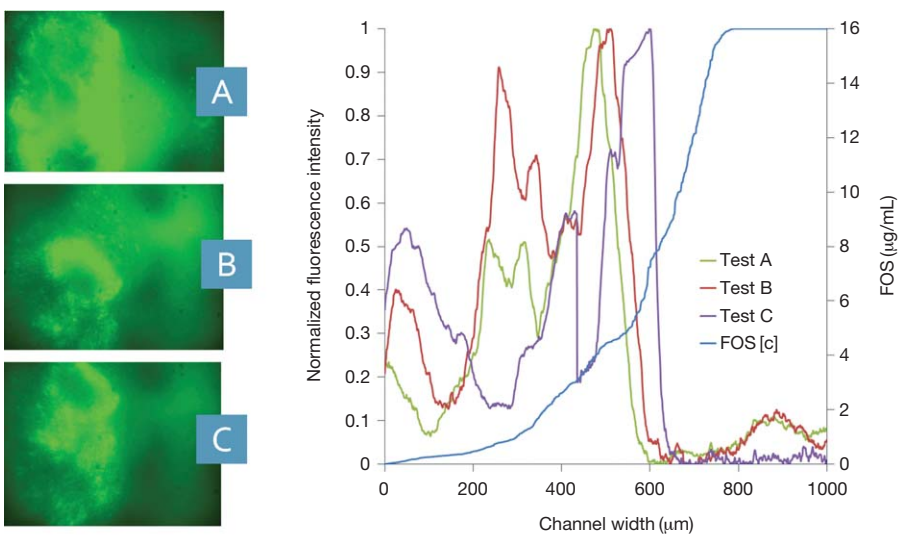

Figure 3. Validation of results at across three subsequent experiments. Results indicate that the concentration of fosfomycin to remediate biofilm-embedded cells of MRSP A12 is 8.1±0.9 μg/mL.

Utility of microfluidic assay

The utility of this microfluidic assay could be adjusted to evaluate the effectiveness of therapies over time as a function of biofilm accumulation. Using the MPA protocols, preliminary experiments passed 100 μg/mL of fosfomycin through the inlets of the mixing module, and observational images were taken over 4 hours. In relating the change in surface coverage area (by function of average fluorescence intensity across the channel) over time, it was noted that appreciable differences in biofilm adherence were observed by 4 hours of incubation in comparison to a control test (double-deionized water added to the inlets for 4 hours with images taken at similar time points). This measure adds another level to the assessment of the chosen therapy. However, it cannot be directly related to that of pharmacokinetic and pharmacodynamic studies, as the antimicrobial is applied directly to the source in consistent concentrations.

Limitations

Adopting microfluidic assays for antimicrobial testing over current methodologies would necessitate that a few issues are considered. One of the major aspects that need to be more carefully examined involves the differences in the adherence capabilities of specific biofilm-producing isolates of MRSP, especially when they are examined in such a dynamic environment. Differences in adherence strength would consequently affect the relative surface coverage in our device; more adherent strains would provide better coverage and density[17]. We also need to take into account that the biofilm must be clearly distinguished from the background fluorescence intensity. Otherwise it may be difficult to discern true biofilm formation from background. Our experiments are performed at a flow speed that is generally accepted as representative of the actual shear forces that act upon biofilm cells during SSIs. Fluid flow rates would directly influence the stability of the gradient over the length of the micro-channel and likely the ability of MRSP to form biofilms and the subsequent structure of the resultant biofilms[18,19]. It is generally accepted that an increase in flow rate strengthens the adherence and the biomechanical properties of cells that remain attached under higher shear stress[24]. The state of the art of convective type of gradient generator and the underlying mass transfer principles has been summarized by Toh *et al*[25]. Hydrodynamic Shear stress and mass transport significantly influence the quality of the biofilm grown in flowing liquid systems such as the developed gradient generator microfluidic platform[15,24]. Flow rate of 200 μL per hour produced a maximum shear stress of 0.27 dyne cm^{-2} on fibroblast cell surface[26], while a flow rate of 100 nL per minute produced a shear stress of 0.055 dyne cm^{-2} on dermal fibroblast cells[27]. The physiological shear stress conditions experienced by pathogenic bacterial biofilms during the course of infection typically range between 4 to 50 dyne cm^{-2}, while critical shear stress for biofilm to survive along blood vessels[28,29] are usually less than 1 dyne cm^{-2}. Hence, the selected flow rate for our experiments provided conditions for the biofilm to survive in a wound like setting. Increased flow rate at a factor of 10 significantly altered the MRSP biofilm clusters (data not shown) possibly due to cellular responses due to shear. Further investigations on the mechanics of detachment and debridement of biofilms under various flow rates due to hydrodynamic shear are warranted.

MRSP biofilm infections are often detected in veterinary out-patients, and hence this delayed detection may allow the biofilm to reach a more mature stage characterized by stronger levels of adherence among the bacterial cells and between the bacterial cells and the tissue. Therefore, experiments examining the effect

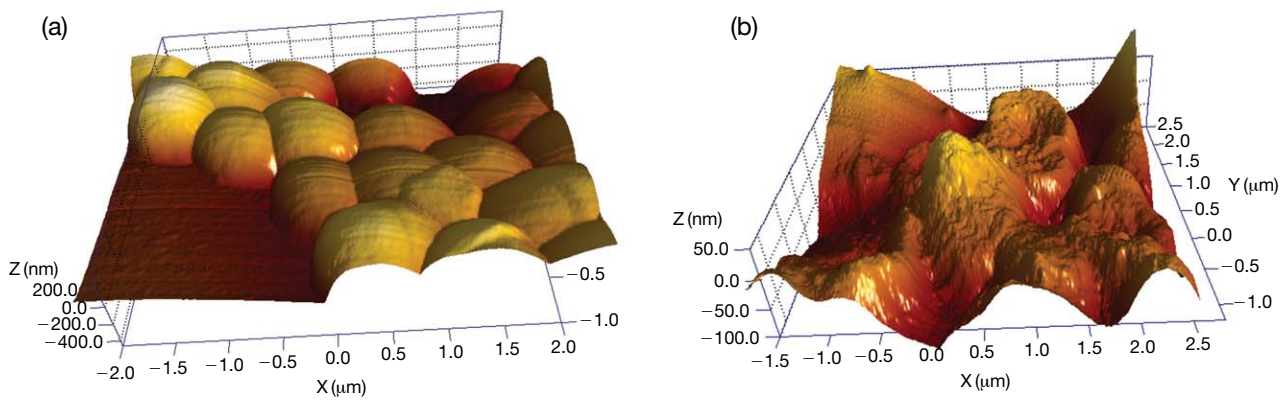

Figure 4. Atomic force microscopy images of MRSP A12 strain (a) control and (b) fosfomycin-treated biofilms.

of fosfomycin on more mature biofilm populations are warranted, potentially also examining other strains of MRSP that may be more or less adherent and/or examining the effects of multi-species infection. Overall, such investigations may aid in the assessment of the clinical relevance regarding the impact of fosfomycin in the remediation of MRSP biofilms in a more realistic setting than other currently implemented models.

AFM analysis

The ability of fosfomycin to kill or inhibit MRSP biofilm was assessed using atomic force microscopy experiments[20]. As characterized by AFM, the sizes of the MRSP cells were in the range of 800 nm to 1000 nm (Figure 4a). MRSP cell surface was found to shrink upon exposure to fosfomycin until the bacteria disappeared from the mica substrate. The external stress caused by the low molecular weight fosfomycin disrupted the cell wall of MRSP (Figure 4b) through membrane damage, followed by the complete disappearance of the substrate surface. The untreated control sample *staphylococci* remained adherent longer on the surface, suggesting that fosfomycin is an effective antibactericidal candidate.

Conclusions

This study developed and demonstrated the potential application of an assay for evaluating varying concentrations of antimicrobial therapy on established MRSP biofilms along a micro-channel. Thus, our technique allows for the rapid analysis of potential remediation therapies directed towards immune and antimicrobial resistant SSIs. Our real time observations of the reduction in the surface coverage area of biofilms inside the microfluidic channel confirmed that fosfomycin is an effective antibacterial agent against MRSP in SSIs. Furthermore, we demonstrated that the microfluidic based investigation of biofilms is an efficient way to study these infectious diseases, although further investigations need to be initiated.

Materials and Methods

Microfluidic passive flow devices

The aim of the experimental procedure is to apply multiple concentrations of antibacterial therapy (fosfomycin) to an established MRSP biofilm along a microchannel. Therefore, we make use of the principles of passive mixing in order to create a gradient-forming device. The key feature in our microfluidic platform is to ensure the presence of laminar flow using syringe pumps. This allows for the generation of spatio-temporal steady gradients between two or more solutions in straight channels, where molecular diffusion-based mixing is dominant. Hence, by combining a gradient generator and a straight main channel (where MRSP biofilms are present), we are able to produce a predictable steady gradient of 2 solutions. Figure 5 schematically depicts our microfluidic device, which consists of a gradient mixing module (20 μm depth × 100 μm width × 18750 μm total length) and the observation module (20 μm depth × 1000 μm width × 12000 μm length). The width of the bacteria inlet channel is 50 μm. The main channel is washed, and a gradient is formed with fosfomycin (red) and PBS (blue) added

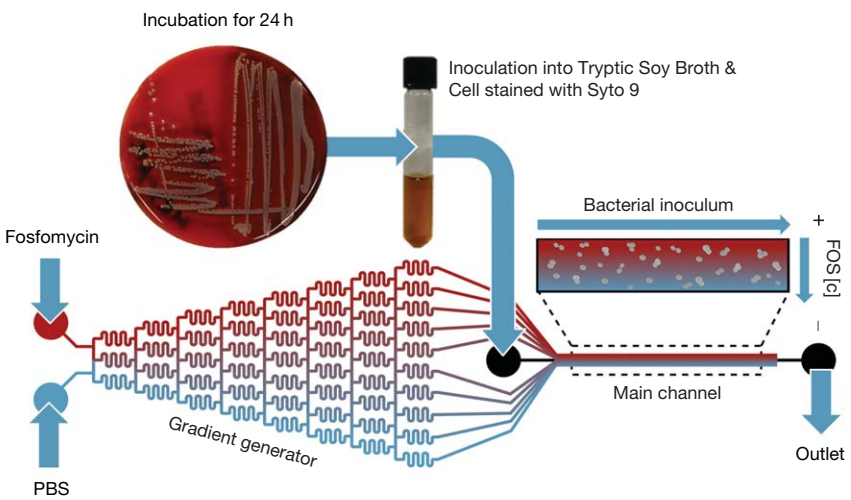

Figure 5. Schematic diagram of the experimental set up of the microfluidic gradient generator device for antimicrobial testing of fosfomycin against MRSP biofilms.

at the inlets to treat the biofilm in the main channel.

Device design and fabrication

The microfluidic template was fabricated and ultimately etched onto a thick photoresist wafer by using e-beam photolithography. This process involves the removal of selected parts of a polymer on a substratum through UV radiation, which allows for minimum feature sizes ranging from a few to hundreds of microns with high-aspect ratios. Consequently, a negative copy of the device was created by pouring an organic silicone polymer, PDMS, over the top of the SU-8 (50). The compound was then solidified by introducing a curing agent that catalyzes the formation of long polymer chains. Finally, the PDMS devices were cut and bonded to a glass cover slip in order to form 0.75 mm diameter inlet and outlet holes.

MRSP inoculum preparation

A MRSP clinical isolate was sub-cultured on Columbia agar plates with 5% sheep blood for 24 h at 35°C. Cultured bacteria were then used to inoculate 5 mL trypic soy broth plus glucose (TSB-G) tubes at a 0.5 McFarland standard (~10^8 CFU/mL) (Sigma-Aldrich Ltd, Canada) and cultured again at 35°C and 140 rpm for 4 h to reach mid-log phase (OD_{600}=0.3). This second growth period was used to encourage cellular adhesion in the main channel of the microfluidic device upon application. The bacterial suspension was then concentrated into 1 mL by centrifugation at 5000 rpm for 5 minutes and washing with PBS for a total 3 wash/spin steps; after the final wash, the cells were suspended in 1 mL PBS. Before application into the main channel of the device, the cells were stained with SYTO 9 dye from a LIVE/DEAD® BacLight™ bacterial viability kit (Molecular Probes, Invitrogen Corp., Carlbad, CA) based on the manufacturer's recommendations.

Biofilm production

Epidemiologically unrelated MRSP isolates from dogs in Canada were obtained from clinical samples submitted to the University of Guelph, Ontario Veterinary College. A biofilm-forming strain of MRSP (A12) that is highly adherent was chosen[16,20] and sub-cultured on blood-agar plates prior to inoculation into TSB-G. Consequently, to facilitate the growth of the biofilm, the inoculated media was pumped with a Harvard Apparatus pump 11 (Harvard Apparatus, Harvard, MA) into the microfluidic device at a rate of 10 μL/h over 24 h and were grown in the dark prior to microscopy to prevent photo-bleaching.

AFM imaging

MRSP A12 cells were imaged in air with a tapping mode Agilent AFM System Model 5500 microscopy (Agilent Technologies, Chandler, AZ) before and after being treated with fosfomycin. AFM height, amplitude, and phase images were obtained in AC mode (tapping mode) on the air-dried mica substrates. A triangular Si cantilever tip (Bruker AFM Probes, Camarilla, CA) with a spring constant of 0.35 N/m and a resonance frequency of 18 kHz was used. A scan speed of 0.7-1.5 Hz was set and resulted in a final resolution of 512 by 512 pixels.

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