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Comparative evaluation of biofilm disinfectant efficacy tests

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Abstract

Regulatory agencies are receiving registration applications for unprecedented, antibiofilm label claims for disinfectants. Reliable, practical, and relevant laboratory biofilm test methods are required to support such claims. This investigation describes the influence of fluid dynamics on the relevancy of a laboratory test. Several disinfectant formulations were tested using three different biofilm testing systems run side-by-side: the CDC biofilm reactor system that created turbulent flow (Reynolds number between 800 and 1850), the drip flow biofilm reactor system that created slow laminar flow (Reynolds number between 12 and 20), and the static biofilm system that involved no fluid flow. Each comparative experiment also included a dried surface carrier test and a dried biofilm test. All five disinfectant tests used glass coupons and followed the same steps for treatment, neutralization, viable cell counting, and calculating the log reduction (LR). Three different disinfectants, chlorine, a quaternary ammonium compound, and a phenolic, were each applied at two concentrations. Experiments were conducted separately with *Pseudomonas aeruginosa* and *Staphylococcus aureus* and every experiment was independently repeated. The results showed that biofilm grown in the CDC reactor produced the smallest LR, the static biofilm produced the largest LR, and biofilm grown in the drip flow reactor produced an intermediate LR. The differences were large enough to be of practical importance. The dried surface test often produced a significantly higher LR than the tests against hydrated or dried biofilm. The dried biofilm test produced LR values similar to those for the corresponding hydrated biofilm test. These results show that the efficacy of a disinfectant must be measured by using a laboratory method where biofilm is grown under fluid flow conditions similar to the environment where the disinfectant will be applied.

Keywords: Antimicrobials; Biofilm reactor; Fluid shear; Regulatory data; Relevant test

1. Introduction

Biofilm bacteria live in a self-organized, cooperative community of microorganisms attached to surfaces, interfaces, or each other, embedded in a matrix of extracellular polymeric substances of microbial origin, and exhibit altered phenotypes with respect to growth rate and gene transcription (Boles et al., 2004; Donlan and Costerton, 2002; Stoodley et al., 2002). Biofilms are prevalent in moist or aqueous environments, even if the surfaces are intermittently dehydrated. Bacteria predominantly exist as biofilm (Costerton et al., 1978; Costerton, 2004; Donlan and Costerton, 2002). Biofilm bacteria are different from their planktonic counterparts (Loo et al., 2000; Sauer et al., 2002; Sternberg et al., 1999).

Biofilm bacteria are notoriously tolerant to conventional chemical disinfectants (Donlan and Costerton, 2002; Stewart et al., 2000). These high tolerances may be caused by slow diffusion through the extracellular polymeric substance matrices, the existence of persister cells, development of resistant phenotypes, and adaptations to micro-environments (Spoering and Lewis, 2001; Stewart et al., 2000). Because detached biofilm clumps retain this increased resistance (Fux et al., 2004) and may contain enough bacteria to give an infective dose (Wilson et al., 2004), biofilm bacteria represent a potential health risk (Armon et al., 1997; Murga et al., 2001).

For these reasons, there is a recognized need for laboratory methods for testing the efficacy of chemical disinfectants against biofilm bacteria. The standard methods used to show

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potential antibiofilm activity of chemical disinfectants often employ the use of planktonic cells that have been dried on a hard surface carrier (ASTM International, 2003). However, a test against actual biofilm bacteria is required for relevancy to real-world applications (Bloomfield and Sims, 1996; Costerton, 2004; Costerton and Stewart, 2001; van Klingeren et al., 1998). Not only must a biofilm disinfection test method include all the biological, chemical, and analytical components of conventional suspension or dried surface tests, but the method also requires some engineered apparatus, such as a biofilm reactor, for growing a reproducible biofilm. Moreover, the laboratory biofilm should be grown so that it possesses the key attributes of the naturally-occurring biofilm where the disinfectant will be applied.

Fluid dynamics are an important consideration when designing a reactor to grow a relevant biofilm (Purevdorj and Stoodley, 2004). A biofilm will self-assemble into a characteristic architecture that depends upon the fluid shear conditions under which it grew. For example, biofilms formed under high shear, turbulent flow, are stronger, more stable, and more strongly attached than their low shear, laminar flow counterparts (Pereira et al., 2002; Purevdorj et al., 2002; Vieira et al., 1993). Biofilms grown in turbulent flow conditions have a greater mass, physiological activity, and total protein than biofilms grown in laminar flow (Simões et al., 2003a,b). Biofilms grown in turbulent flow are more dense than the fluffy biofilms grown in laminar flow (Pereira et al., 2002). It would be prudent to engineer fluid dynamics within the biofilm growth reactor that emulate the fluid dynamics in the target environment (Blanchard et al., 1998; Eginton et al., 1998; Simões et al., 2003a,b, 2005).

A variety of reactors and growth systems have been used successfully for research and/or disinfectant testing purposes (e.g., Ceri et al., 1999; Characklis, 1990; Charaf et al., 1999; Gilbert et al., 1998; Goeres et al., 2005; Kharazmi et al., 1999; Luppens et al., 2002; Pitts et al., 2003; Stoodley and Warwood, 2003; Wilson, 1999; Zelver et al., 1999). For the development and official registration of commercial disinfectants against biofilm bacteria, standardized laboratory reactors and associated standard operating procedures are required. The standardization process for biofilm tests has just begun. At present, only one biofilm reactor and operating procedure has been approved by the USA standard setting organization American Society for Testing and Materials International (ASTM), method #E 2196-02 (ASTM International, 2002). Tests of antibiofilm efficacy are based predominantly on ad hoc disinfectant testing methods.

The main goal of this study was to compare the efficacy results for three biofilm disinfectant tests, where each test utilized a different type of reactor fluid dynamics: turbulent flow, laminar flow, and no flow. Also included in the comparative study were disinfectant challenge tests against dried biofilm and a current hard surface carrier test method. Each biofilm growth reactor and associated test method chosen for this comparative study has potential for standardization, and consequently is a candidate method for the development, testing and registration of antibiofilm disinfectants. Therefore, the results include the statistical characteristics, such as the mean viable cell density on control carriers and the repeatability SD for LR values, for each of the individual tests.

2. Materials and methods

2.1. Bacterial species/strains and inoculum preparation

Pseudomonas aeruginosa ATCC 15442 were grown in 300 mg tryptic soy broth (TSB) l^{-1} and *Staphylococcus aureus* ATCC 6538 were grown in 30 g TSB l^{-1}. Both were incubated for 18–24 h in a 37 °C shaker. Bacteria were transferred no more than 5 times from the original culture stock.

2.2. Coupons and cleaning procedure

All five test methods used borosilicate glass coupons, which were disks having a diameter of 1.27 cm and a height of 0.4 cm (BioSurface Technologies, Corp., Bozeman, MT). Prior to use, the coupons were visibly inspected and discarded if flawed. They were cleaned according to ASTM E2196-02 (ASTM International, 2002).

2.3. CDC biofilm reactor method (CDC)

A high shear biofilm was grown in the CDC biofilm reactor (model CBR 90-1, BioSurface Technologies Corp., Bozeman, MT) as described by Goeres et al. (2005) and shown in Fig. 1. A 1 ml volume of *P. aeruginosa* or *S. aureus* inoculum was inoculated into the reactor containing 300 mg TSB l^{-1} or 30 g TSB l^{-1}, respectively. The reactor stood on a digital stir plate set at 180 rpm for *P. aeruginosa* or 125 rpm for *S. aureus*. The digital stir plate controlled the rotating baffle, which is that component of the reactor that produced fluid shear on the coupon surfaces. *P. aeruginosa* biofilm was grown at 23±1 °C in batch conditions for 24 h, followed by a continuous flow of 300 mg TSB l^{-1} at a rate of 11.45 ml min^{-1} for an additional 24 h. The *S. aureus* biofilm was grown at 35±2 °C in batch conditions for 24 h, followed by a continuous flow of 3 g TSB l^{-1} at a rate of 11.47 ml min^{-1} for an additional 24 h.

2.4. Drip flow biofilm reactor method (DF)

A low shear biofilm was grown in a modified drip flow reactor (model DF 202, BioSurface Technologies Corp., Bozeman, MT), as shown in Fig. 1. The previously described reactor system (Stewart et al., 2001) was modified to accommodate rubber sheeting machined to hold one coupon in each of the four channels. Each channel, containing 20 ml of 300 mg TSB l^{-1} or 30 g TSB l^{-1}, was inoculated with 1 ml of the *P. aeruginosa* or *S. aureus* inoculum, respectively. The *P. aeruginosa* biofilm was grown at 23±1 °C and the *S. aureus* biofilm was grown at 35±2 °C in batch for 24 h. Continuous flow was started immediately by placing the reactor on a stand with a 10 °C angle and pumping 300 mg TSB l^{-1} through at a flow rate of 0.82 ml min^{-1} per channel for *P. aeruginosa* or 3 g