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ULTRAVIOLET DISINFECTION EFFICACY TEST METHOD USING BACTERIA MONOLAYERS

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Abstract

Monolayers of bacterial cells of Staphylococcus aureus and Pseudomonas aeruginosa were inoculated on glass slide carriers using an automated inoculum spray deposition system. The use of bacterial monolayers allows for control of critical variables for testing and verification of light-based disinfection technologies. This approach avoids the variability associated with manual inoculation and high inoculum titers, which can engender clustering of cells and the associated photoprotection that clustering incurs. The use of glass slide carriers avoids problems caused by irregular microscopic surface features, which can impact the efficacy evaluation of light-based disinfection technologies. Scanning electron micrographic (SEM) imaging was used to verify the surface topography and the presence of monolayers. The spray deposition method produced a mean density of $>10^6$ colony forming units (CFU) per carrier. The inoculated carriers were exposed to ultraviolet light for 120 s from a focused multivector ultraviolet (FMUV) light system. A mean log CFU reduction of 4.8 was achieved for *S. aureus* ($p < 0.0001$). A mean log CFU reduction of 5.1 was achieved for *P. aeruginosa* ($p < 0.0001$). The test method presented herein will facilitate increased accuracy in the measurement of ultraviolet susceptibility rate constants.

Introduction

Ultraviolet (UV) disinfection technologies have seen accelerated implementation in healthcare and other industries in response to the spread of multidrug resistant organisms (MDROs), especially in the context of the current COVID-19 pandemic. Increased interest in efficacy testing has highlighted the absence of UV laboratory test methods. As investigators seek to achieve ever higher performance levels, certain confounding factors arise as a barrier to precision testing beyond 3–6 logs of reduction. Currently, the only standard addressing UV disinfection of hard surfaces is ASTM E3135 (ASTM, 2018), but this standard employs steel carriers and manual inoculant deposition, methods which are shown herein to introduce confounding factors and t limit the accuracy and reproducibility of the results.

Various challenges have confronted investigators seeking to develop accurate and reproducible protocols for testing the efficacy of ultraviolet disinfectant systems. Traditional microbiological methods for measuring UV disinfection rates suffer from variability with the primary problems being due to 1) the inoculation method and solution concentration, 2) the substrate and substrate reflectivity, and 3) solution contaminants. Varying the inoculant concentration and the carrier surface concentration can directly impact germicidal performance data (Furness, 1969; Johnston et al., 2000; Cadnum et al., 2016; Wong et al., 2016). Inocula above 10^6 CFU/ml have been shown to produce clumping of cells (Levy et al., 2011). The inoculant concentration in UV experiments has long been noted to influence tailing in survival curves, which manifests as an increase in UV resistance at extended UV dosages (Furness, 1969; Gomez-Lopez et al., 2005; Boyce et al., 2011). Clumping of cells can offer photoprotection and produce tails in the survival curve (Kowalski et al., 2020; Doughty et al., 2021). As investigators increasingly target log reductions of >3–4 logs, and often 6 logs or more, manual deposition of inoculant may pose limits to accuracy. Manual spreading of high inoculum concentrations over larger areas has been shown to improve results but this introduces an additional variable that is not easily controlled by protocols (Cadnum et al., 2016). A more effective solution is to eliminate the causes of variability and/or minimize their influence on experimental error.

The tailing of survival curves is an artifact of the preparation methods and is not an intrinsic property of the microorganisms (Kowalski et al., 2020). The use of high concentrations of test bacteria can produce clusters of cells in which the innermost cells are partially shielded against UV radiation (Kowalski et al., 2020). Manual inoculation of *C. difficile* spores resulted in clumping of spores, a fact attested to by the SEM photographs that were taken. Fig. 1 (Left) shows an example of clusters that formed after manual inoculation of a sample of *Clostridioides difficile* on stainless steel. The protection from UV exposure afforded by the clustering of cells manifests itself as a tail in the survival curve at higher dosages. This tail is variable depending on the degree of clustering and it is unpredictable. The appearance of a tail in the survival curve can distort the results (i.e. D90 measurement) and limits the precision with which the UV rate constant can be measured (i.e. a tail at 3 logs reduction limits the precision to 3 decimal places).

The use of monolayers of cells will produce a single stage of exponential decay without a tail (Raguse et al., 2016; Kowalski et al., 2020). When there is only a single stage of decay, extreme accuracy is possible, both in measuring efficacy and in establishment of precise UV rate constants for any given pathogen. Deposition of monolayers of *S. aureus* and *P. aeruginosa* resulted in no clumping as verified by SEM photography.

The substrate can confound the results of UV efficacy testing, and studies show wide variation in the response of microbes when irradiated on different materials such as plastic, formica, steel and glass (McKenzie et al., 2013; Cadnum et al., 2016). The most commonly used substrate materials are glass and stainless steel. Fig. 2 illustrates the surface roughness of these materials at a magnitude which shows *C. difficile* spores. Note in the right photo that the surface asperities are large enough to provide some shadowing from ultraviolet light and that some cavities are large enough to enclose entire cells. The glass surface (Left photo), however, is remarkably smooth at this magnification and provides no shielding effects

Fig. 3 shows two examples of contaminants in the inoculation. Some contaminants like Tween-80 (Left image) can form a sludge-like residue that may interfere with UV treatment and that may also cause clumping. The presence of salts in the dried sample may result in large-scale (i.e. 50 µm) evaporative rosettes or other structures that may interfere with the UV treatment process. A single washing step as specified in EPA standards like MB-06-09 or MB-31-03 is inadequate for UV testing purposes. Additional washing steps are needed to ensure the final solution is free of contaminants.

Material UV reflectivity is another material factor that can distort the measured level of irradiance. The light that reflects backwards from the carrier surface increases the local absorbed UV dose, rendering the light irradiance measurement inaccurate, especially for UV rate constant determination. A review of candidate materials indicates that glass is a practical solution to the problem. Glass has among the lowest UV reflectivity of any material (6%), which is far less reflective than stainless steel (28–37%).

Previous studies have created monolayers of bacterial spores for use in ultraviolet testing and creation of biodosimeters (Raguse et al., 2016; Lee et al., 2011). No previous studies have tested ultraviolet germicidal performance with monolayers of vegetative bacteria. The novel method presented here is a feasible test method for accurately evaluating the efficacy of ultraviolet germicidal irradiation technologies and thereby providing a rigidly controlled basis for comparative evaluation. The monolayer method also enables measurement of ultraviolet rate constants with greater precision and reproducibility than was previously possible.

Section Snippets

Materials and methods

Bacterial samples were inoculated onto glass carriers at a certified third party laboratory using an automated inoculum spray deposition system after which the samples were irradiated for 120 s and enumerated. SEM images were used to verify the monolayers.…

Results

Table 1 summarizes the data for *Staphylococcus aureus*. For the lowest dilution of the Replicate Test #3 carrier, in which there was no growth, a value of 0.5 CFU/Carrier was substituted as per the data analysis methods of typical standards (EPA, 2014c). This substitution represents one-half of the lower Limit of Detection (LOD) of 1 CFU per plate and results in a LOD of 9 CFU/carrier for the Test Replicate #3 carrier. Using a value of LOD ÷ 2 is conservative in this case and is one of several…

Discussion

This study demonstrates the feasibility of using monolayer deposition of bacterial cells in a UV efficacy test. The methods developed here have addressed the issues that have confronted other investigators seeking to develop standardized efficacy testing and these methods may provide considerable improvement in accuracy and reproducibility. The test conducted according to this method measured CFU reductions for *S. aureus* in the range of 99.9978% - 99.9995% and for *P. aeruginosa* the range of…

Declaration of Competing Interest

The authors have no conflicts of interest to declare.…

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