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Evaluation of Petrifilm™ Aerobic Count plates as an equivalent alternative to drop plating on R2A agar plates in a biofilm disinfectant efficacy test

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Abstract

This paper compares Petrifilm™ Aerobic Count (AC) plates to drop plating on R2A agar plates as an alternative method for biofilm bacteria enumeration after application of a disinfectant. A *Pseudomonas aeruginosa* biofilm was grown in a Centers for Disease Control and Prevention (CDC) biofilm reactor (ASTM E2562) and treated with 123 ppm sodium hypochlorite (as free chlorine) according to the Single Tube Method (ASTM E2871). Aliquots from the same dilution tubes were plated on Petrifilm™ AC plates and drop plated on R2A agar plates. The Petrifilm™ AC and R2A plates were incubated for 48 and 24 hours, respectively, at 36 ± 1 °C. After 9 experimental runs performed by 2 technicians, the mean difference in biofilm log densities ($LD = \log_{10}(\text{CFU}/\text{cm}^2)$) between the two methods for control coupons, treated coupons, and log reduction (LR) was 0.052 ($p=0.451$), -0.102 ($p=0.303$), and 0.152 ($p=0.313$). Equivalence testing was used to assess equivalence of the two plating methods. The 90% confidence intervals for the difference in control and treated mean LDs between methods were (-0.065, 0.170) and (-0.270, 0.064), both of which fall within a (-0.5, +0.5) equivalence criterion. The 90% confidence interval for the mean LR difference, (-0.113, 0.420), also falls within this equivalence criterion. Thus, Petrifilm™ AC plates were shown to be statistically equivalent to drop plating on R2A agar for the determination of control LDs, treated LDs, and LR values in an anti-biofilm efficacy test. These are the first published results that establish equivalency to a traditional plate counting technique for biofilms, and for a disinfectant assay.

Keywords: Petrifilm; biofilm; CDC reactor; efficacy testing; equivalence testing

Introduction

A critical aspect of microbiological methods is the technique used to enumerate the microorganisms. Recent advancements in technology have begun to yield faster, more efficient methods for the quantification of cells. Methods such as fluorescence [23], bioluminescence [3], flow cytometry [16], autofluorescence [7], real-time polymerase chain reaction [11], and infrared spectroscopy [26] are now being used to quickly quantify bacteria. Without staining the cells or coupling methods, the techniques listed above are unable, individually, to quantify cells based upon viability [4, 16, 23, 27]. The viable plate count method is the most common method used to quantify viable, culturable bacterial cells, although it can be time-consuming, labor intensive, and may select against certain organisms [7, 21, 22].

Petrifilm™ Aerobic Count (AC) plates (3M™, Saint Paul, MN) is a ready-to-use product for the enumeration of viable bacteria. Each Petrifilm™ AC plate is a two-piece film containing culture media, a cold-soluble gelling agent, and a tetrazolium indicator. The use of Petrifilm™ AC plates eliminates the need to prepare Petri plates and saves space [18]. Stacked, 20 Petrifilm™ AC plates take up about the same space as four Petri plates. Studies have been conducted comparing the effectiveness of using Petrifilm™ AC plates to traditional plating methods (e.g. drop, pour, and spread plating) in the food industry [8, 14, 20]. These studies revealed a high degree of association between traditional methods and Petrifilm™ AC plates. One novel contribution of our paper is to compare plating techniques for a biofilm disinfectant efficacy assay.

Viable plate counts are an important component of disinfectant efficacy testing. Environmental Protection Agency (EPA) product testing guidelines for disinfectants require viable plate counts for bacterial enumeration [28], ensuring the continued use of the viable plate count assay. Disinfectant efficacy against biofilms is of particular concern, given that biofilm communities have a demonstrated tolerance to antimicrobial agents [9, 13, 15]. ASTM (formerly known as the American Society for Testing and Materials) Method E2562, “Standard Test Method for Quantification of a *Pseudomonas aeruginosa* Biofilm Grown with High Shear and Continuous Flow using a Centers for Disease Control and Prevention (CDC) Biofilm Reactor” is an approved, standardized test method for growing a reproducible biofilm [5]. This biofilm can then be used for disinfectant efficacy testing by following

ASTM Method E2871, “Standard Test Method for Evaluating Disinfectant Efficacy against *Pseudomonas aeruginosa* Biofilm Grown in CDC Biofilm Reactor using Single Tube Method” [6]. Both methods require viable plate counts using an “accepted plating technique” such as spread, drop, pour, or spiral plating.

The goal of this project was to determine if drop plating on R2A agar versus plating on Petrifilm™ AC plates produces statistically equivalent results for the Single Tube Method. Statistical equivalence tests were used rather than conventional hypothesis testing. Failure of a hypothesis test to find a significant difference between two methods does not necessarily suggest equivalence; instead one can only conclude that there is a lack of evidence to suggest that the methods are different. For example, if two test methods are compared that consistently give different results, but if one method is highly variable, then a traditional hypothesis test would likely lead to the conclusion that there is no significant difference between the two methods. A conclusion of equivalence of the two methods in this instance would be inappropriate. Equivalence testing is the appropriate statistical tool in this context because it specifies an acceptable level of variability; if the mean differences between the methods are less than the acceptable level with a high degree of confidence, only then can equivalence be concluded.

Methods

Experimental Design

Each experiment consisted of growing a *P. aeruginosa* biofilm in a Centers for Disease Control (CDC) biofilm reactor [5], treating the biofilm with sodium hypochlorite according to the Single Tube method [6], and enumerating viable bacteria using R2A agar and Petrifilm™ AC plates both inoculated from the same dilution tube. Two technicians each conducted their own experiments side-by-side on the same days, including sampling on the same day at the same time. Five experiments were conducted by each technician, but one experiment by one technician was invalidated due to a failed inoculation. Three control and three treated coupons were sampled per experiment and each plating method was performed in duplicate for each type of coupon.

Bacterial strains and inoculum preparation

Pseudomonas aeruginosa (ATCC 15442) frozen stocks were streaked for isolation on R2A agar plates. Three to five isolated colonies were added to 100 mL of 300 mg/L tryptic soy broth (TSB). The inoculum was placed in a $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$ shaker/incubator at 125 rpm for 24 hours.

CDC biofilm reactor method

A biofilm was grown under high shear conditions on borosilicate glass coupons in a CDC biofilm reactor (BioSurface Technologies, Corp., Bozeman, MT) according to ASTM E2562 [5]. In summary, 1 mL of 10^8 colony forming units (CFU)/mL *P. aeruginosa* inoculum was aseptically pipetted into a sterile CDC reactor containing approximately 500 mL of 300 mg/L TSB with a clamped effluent line. The reactor sat on a digital stir plate set to 125 rpm. The biofilm was grown in batch conditions at $20^{\circ} \pm 1^{\circ}\text{C}$ for 24 hours. After this time, the effluent was unclamped and a continuous nutrient flow of 100 mg TSB/L was supplied for an additional 24 hours. The nutrient flow rate was calculated as the volume of the reactor liquid (with effluent unclamped) divided by a 30 minute residence time for the bacteria. This flow rate, theoretically, washes out planktonic cells while leaving biofilm cells attached to reactor surfaces.

Disinfectant and neutralizer preparation

A 123 ppm solution of sodium hypochlorite as free chlorine was prepared. Free chlorine concentrations were measured spectrophotometrically following the DPD Colorimetric Standard Method 4500-Cl G [2] using Hach free chlorine reagent packets (Cat. No. 1407799, Hach Co., Loveland, CO) and a potassium permanganate standard curve. Treatments were kept at $20 \pm 1^{\circ}\text{C}$ and used within three hours of preparation. The sodium hypochlorite was neutralized with 36 mL of a filter sterilized, 97.2 mg/L solution of sodium thiosulfate in deionized water.

Treatment, removal, and disaggregation of biofilm

After growing a biofilm in the CDC reactor, coupons were removed, rinsed, treated, and sampled according to the Single Tube method [6]. Per the method, the biofilm-covered coupons were placed in 50 mL conical tubes. Four mL of disinfectant was added to each conical tube for a contact time of 10 minutes followed by 36 mL of neutralizing solution. The control coupons were treated with 4 mL of sterile, buffered dilution water for 10 minutes, followed by the addition of 36 mL of the neutralizing solution. The tubes were vortexed (30s, high), sonicated (30s, 45 kHz, 10% power), vortexed, sonicated, and vortexed to remove and disaggregate the biofilm.

Plating methods

Following removal and disaggregation, biofilm samples were serially diluted (10^0 to 10^{-7}) in dilution tubes filled with 9 mL of sterile, buffered dilution water. For the drop plate method, 0.1 mL aliquots were drawn up from each dilution tube using an automatic pipette. Fifty μ L of the total volume was pipetted onto two R2A plates, each receiving 5, 10 μ L drops [12]. To plate a sample on the Petrifilm™ AC plate, the top sheet was lifted, 1 mL of sample was pipetted onto the surface, the top sheet was lowered, the provided spreader was placed with the recessed side down over the sample on the top sheet of the Petrifilm™ AC plate, and pressure was gently applied by pressing on the top of the spreader, which spread the liquid over a uniform surface area [1]. All plating was done in duplicate. All samples were plated on R2A agar before being plated on Petrifilm™ AC plates. Petrifilm™ and R2A plates were incubated at 36 ± 1 °C for 48 and 24 hours, respectively.

Statistical Analysis

The lowest countable dilution was enumerated for each plating method to determine biofilm density. Two Petrifilm™ AC plates from a single dilution containing 30-300 colonies each were enumerated and the counts averaged. For the drop plates, the dilution with drops containing 3-30 colonies/drop was enumerated. The final CFU value for the drop plates was an average of all ten drops (five per duplicate plate). Equation 1 uses these values to calculate the average log biofilm density (LD) grown on each CDC reactor coupon, reported as \log_{10}

(CFU/cm²). In Equation 1, the volume plated for Petrifilm™ AC and R2A plates was 1 mL and 0.01 mL, respectively.

$$\log_{10} \left(\frac{CFU}{cm^2} \right) = \log_{10} \left(\frac{\text{average CFU}}{\text{volume plated (mL)}} \times \frac{\text{volume in original sample tube (mL)}}{\text{area of coupon (cm}^2\text{)}} \times \text{Dilution Factor} \right) \quad (1)$$

The log reduction (LR) was calculated to determine the efficacy of the sodium hypochlorite treatment. The LR value for each experiment is the mean LD value of the treated coupons subtracted from the mean LD value of the control coupons [29]. LRs were calculated for each plating method, which yielded a Petrifilm™ AC LR and R2A LR for each experiment.

The plating methods were paired in order to maximize statistical power, meaning that the aliquot plated for each method came from the same dilution tube. Thus, a difference in observed LDs between methods was calculated for each coupon. These LD differences form the basis for comparing the enumeration methods with respect to the untreated control mean LDs and the treated mean LDs. The mean LRs for the two methods were compared considering the difference in LRs for each experiment.

Descriptive statistics were generated for each of the plating methods by fitting an ANOVA to each of the following responses: the control LDs per coupon; the treated LDs per coupon; and the LRs per experiment. An ANOVA was fit to each of the control and treated LDs separately, with crossed random effects due to technician and experiment. For the LRs, the ANOVA had a single random effect due to technician. The ANOVA provided variance estimates for within-experiment, among-experiment, and between-technician sources. The repeatability standard deviation (SD) was calculated by taking the square root of the sum of the positive variance components: within-experiment divided by the number of coupons (3) in each experiment, among-experiment, and between-technician.

The two plating methods were compared by fitting an ANOVA to each of the following responses: the difference in control LDs per coupon; the difference in treated LDs per coupon; and the difference in LRs per experiment. For the first two responses, crossed random effects were included for technician and experimental day. For the difference in LRs, the ANOVA had a single random effect due to technician. These ANOVAs were used to test for statistically significant differences and statistical equivalence between the two plating methods.

From each of the ANOVAs, 90% confidence intervals (CI) were constructed for the mean difference in the control LDs, the mean difference in the treated LDs, and the mean difference in LR between the two plating methods. To determine statistical equivalence of the two plating methods with 95% confidence, it suffices to show that the 90% CIs for the mean differences were contained in the interval $[-\delta, \delta]$ for a specified value of δ [25]. Consistent with previous work, we chose $\delta=0.5$ [19]. In other words, we considered differences on the average less than $0.5 \log_{10}(\text{CFU}/\text{cm}^2)$ to be negligible and not of practical importance.

The ANOVAs were implemented using the statistical software Minitab® [17]. All statements regarding statistical significance, including equivalence tests, are based on a significance level of 5%.

Results

Control Samples

The control LDs are graphed in Figure 1. Summary statistics for the two methods are displayed in Table 1. The differences in LDs between the methods are graphed in Figure 2. The mean difference in untreated control LDs between the Petrifilm™ AC and the R2A plates across all the experiments was $0.052 \log_{10}(\text{CFU}/\text{cm}^2)$ (Table 2). There was no statistically significant difference of the mean control LDs between the two methods ($p=0.451$). The 90% CI for the mean difference in control LDs between the methods, $(-0.065, 0.170)$, demonstrates that the Petrifilm™ AC plates and drop plating on R2A agar are statistically equivalent methods for enumerating untreated, control *P. aeruginosa* biofilm.

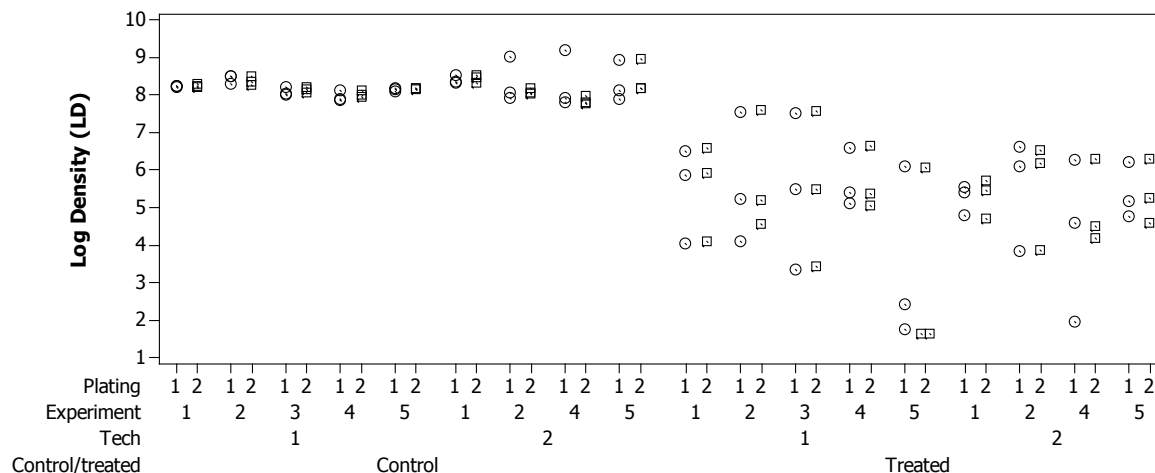


Figure 1 LD values for each plating method in each experiment. Control coupon LDs are displayed on the left and treated coupon LDs are on the right. Each pair of points (3 circles paired with 3 squares) represents the pairing of the Petrifilm™ to R2A agar plates in each experiment. Plating method 1(circles) represents counts from the Petrifilm™ AC plates and plating method 2 (squares) represents the counts from the R2A plates. Each point in the plot is the LD for a single coupon

Treated Samples

The treated LDs are graphed in Figure 1. Summary statistics for the two methods are shown in Table 1. The differences in LDs between the two methods are displayed in Figure 2. The mean difference between the treated LDs for the Petrifilm™ AC plates and R2A drop plates was $-0.102 \log_{10}(\text{CFU}/\text{cm}^2)$. There was no statistically significant difference between the mean LDs reported by the two methods ($p=0.303$). The 90% CI for the mean difference in treated LDs between methods was $(-0.270, 0.064)$, which fits inside the $(-0.5, +0.5)$ equivalence criterion (Fig. 3). This suggests that Petrifilm™ AC and R2A drop plates are statistically equivalent methods for enumerating *P. aeruginosa* biofilm samples treated with sodium hypochlorite and neutralized with sodium thiosulfate.

The LRs reported by the two methods are also displayed in Table 1. The mean difference in LRs between the

methods was 0.152, as shown in Table 2. There was no significant difference in mean LRs between the methods ($p = 0.313$). The 90% CI for the mean difference in LRs, (-0.113, 0.420), satisfies the equivalence criterion and demonstrates that the two methods are statistically equivalent with respect to LRs.

Table 1 Summary statistics for each of the two plating methods. The repeatability SDs are based on 3 control coupons and 3 treated coupons. The percentages of variance due to each of the three contributors to the repeatability standard deviation are also shown.

	Plating Method	Mean LD Log ₁₀ (CFU/cm ²)	Repeatability SD	Variability Sources (%)		
				among- technician	among- experiment	within- experiment
Control	Petrifilm™	8.24	0.213	2	0	98
	R2A	8.19	0.189	0	62	38
Treated	Petrifilm™	5.15	0.930	0	0	100
	R2A	5.26	0.876	0	0	100
Log Reduction	Petrifilm™	3.09	1.04	0	0	100
	R2A	2.93	0.864	0	8	92

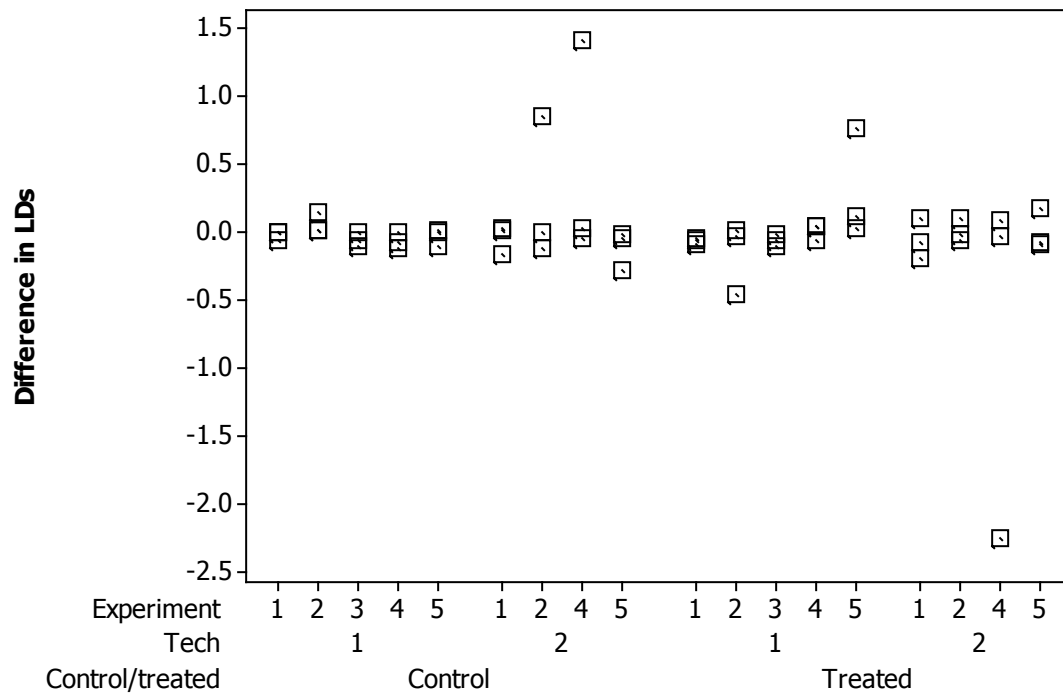


Figure 2 Differences between LDs from each pair of plating methods. Each point represents the difference in LDs between Petrifilm™ and R2A agar when plated with samples from the same dilution by a single technician

Table 2 Statistical comparison of the plating methods. These results show no statistically significant differences between the two methods. Equivalence of the methods was concluded because all three 90% CIs fit within the (-0.5, +0.5) equivalence criterion.

	Mean Difference	Standard Error	P-value	90% CI
Control	0.052	0.068	0.451	(-0.065, 0.170)
Treated	-0.102	0.097	0.303	(-0.270, 0.064)
Log Reduction	0.152	0.139	0.313	(-0.113, 0.420)

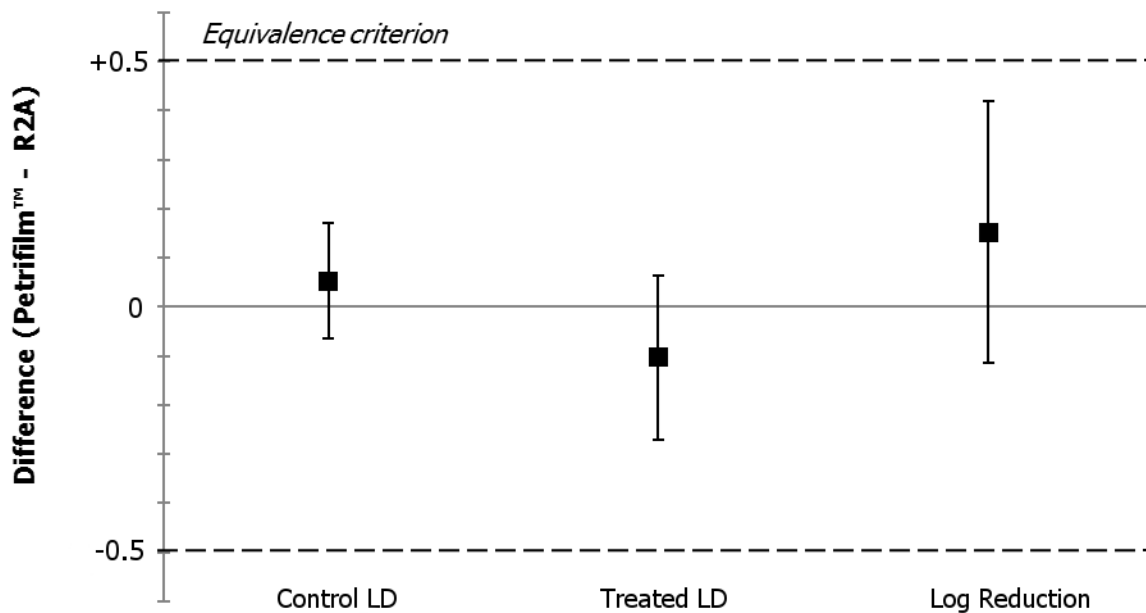


Figure 3 Graphical representation of 90% CIs for the three responses tested. Confidence intervals were constructed based upon the difference in results between plating methods for each response. The CIs fit within the (+0.5,-0.5) equivalence criterion, demonstrating statistical equivalence between methods for these three responses

Discussion

Viable plate count methods remain a necessary component of many microbiological methods used today, despite the increasing development and implementation of novel technologies in the lab for the enumeration of microorganisms. As these new methods are implemented and begin to replace traditional methods, how can one be sure that the methods provide equivalent results? In this paper, we demonstrated how statistical equivalence testing can be used to address this very general and important question.

Our experimental results suggest that Petrifilm™ AC plates provided statistically equivalent results to drop plating on R2A agar in the enumeration of *P. aeruginosa* biofilm grown in the CDC reactor (ASTM E2562) on control coupons, and also on coupons subjected to the Single Tube disinfectant efficacy test method (ASTM E2871). These conclusions of equivalence of the control and treated coupon LDs presume that the two methods are equivalent as long as a mean differences in the LDs as large as 0.5 can be considered negligible. This

assumption was based upon years of practical observations of the random error associated with viable plating techniques using this method. This half-a-log criterion is also consistent with the equivalence criterion of (-0.5, +0.5) used for control LDs in a similar, multi-lab study that compared Petrifilm™ AC plates to spread plating for different standardized, dry surface antimicrobial test methods against *B. subtilis*, *S. aureus*, *P. aeruginosa*, and *S. enteric* [19]. Our study is the first published comparison of biofilms and also of equivalency after application of a disinfectant and neutralizing agent. In our experiments using a standardized biofilm antimicrobial test method (ASTM E2871) against a *P. aeruginosa* biofilm, both the Petrifilm™ AC and R2A plating methods demonstrated similar mean LDs and repeatability SDs across multiple experiments and technicians. It is important to note that both methods showed negligible technician-to-technician contributions to the overall variability for the control LD, treated LD and LR values (Table 1). These results show that, in addition to statistical equivalence of the mean biofilm LDs and LRs, the Petrifilm™ AC plates performed similarly to drop plating on R2A agar with respect to the variability of these responses.

The LR is a typical measure of the antimicrobial efficacy of a disinfectant. Thus, it is the most important response considered in our experiments. As expected, the LRs exhibited increased variability compared to LDs since, by construction, a LR has two sources of variability: one due to the control coupons and a second due to the treated coupons. Accordingly, European guidelines for equivalence testing of handrub efficacy have proposed using (-0.6, +0.6) as an equivalence criterion for LRs [10]. Nonetheless, the methods in our study demonstrated statistically equivalent LRs on the average using the more stringent (-0.5, +0.5) equivalence criterion.

The reasonableness and efficiency of using Petrifilm™ AC plates were qualitatively compared against the drop plate method by the two technicians that ran the experiments. The pre-made nature of the Petrifilm™ AC plates saved the technicians approximately 1.5 hours of plate-prep time for every 100 Petrifilm™ AC plates used. We found the drop plate method to be faster since each plate can be divided into quadrants and used for multiple dilutions, whereas each Petrifilm™ AC plate is only used for a single dilution. It is, however, possible to make the

methods more comparable by plating multiple dilutions on a single Petrifilm™ AC plate, although this was not examined in this research [24]. Petrifilm™ AC plates may be more effective when used in industrial applications where it is more economical to purchase pre-made materials, which helps explain its widespread use in food microbiology.

Regulatory agencies such as the EPA rely on traditional, well established methods and it may be difficult to amend regulatory methods to include Petrifilm™ AC plates without extensive study. The recent collaborative study reported by Nelson et al. showed that Petrifilm™ AC plates were statistically equivalent to spread plating methodologies for the three AOAC (formerly known as the Association of Analytical Chemists) antimicrobial testing methods, but their study did not compare either the LDs of treated coupons or LR values between the plating methods [19]. The results from our study help to provide supporting evidence that Petrifilm™ AC plates are an equivalent alternative to drop plating on R2A agar for the quantification of control and treated biofilm LDs and, most importantly, for the determination of LR values.

Conclusions

We were able to demonstrate that Petrifilm™ AC plates and R2A agar plates were statistically equivalent methods for the enumeration of control coupons with *P. aeruginosa* biofilms grown in the CDC reactor (ASTM E2562). These results extend to biofilm results described in previous literature for planktonic and dried surface tests. Statistical equivalence between the two methods was also demonstrated for *P. aeruginosa* biofilm coupons treated with sodium hypochlorite according to the Single Tube disinfectant efficacy test method (ASTM Method E2871). Most importantly, LR values from the two methods, common measures of disinfectant efficacy, were statistically equivalent as well. These statements of statistical equivalence are based on the assumption that mean differences as large as ± 0.5 are negligible and not of practical importance. These are the first published results that compare Petrifilm to a traditional plating method after the microbes have been treated with a disinfectant.

Technicians also qualitatively assessed the efficiency associated with the Petrifilm™ AC plates and determined that it was faster to drop plate multiple dilutions on R2A agar than to plate single dilutions on Petrifilm™ AC plates. Petrifilm™ AC plates, however, were more efficient in terms of preparation time and space consumption.

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