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RelyOn™ Screening for Efficacy Against *Pseudomonas aeruginosa* Biofilm Using the MBEC Assay

PROJECT NUMBER: CD-032
CD REPORT NUMBER: CDR-172-16

SUMMARY:

RelyOn™ when used at a 1:100 dilution was very efficacious against *Pseudomonas aeruginosa* cells in a 16 to 18-hr biofilm (complete kill, ≥ 5.4 $\log_{10}$ reduction) and demonstrated 100% removal of the biofilm after a 4-hr contact time in an MBEC assay.

PURPOSE:

This study was designed to determine the efficacy of RelyOn™ at 1% against *P. aeruginosa* biofilms using an MBEC assay.

MATERIALS & METHODS:

Test Method: ASTM E2799-12 (approved 1-Apr-2012): Standard Test Method for Testing Disinfectant Efficacy against *Pseudomonas aeruginosa* Biofilm using the MBEC Assay.

Test Bacterium:

- *Pseudomonas aeruginosa* ATCC 15442

Inoculum Prep:

A fresh colony of *P. aeruginosa* was transferred to Tryptic Soy Agar (TSA) and incubated overnight (O/N) at 35°C for 16-18 hr. Then an isolated colony was isolated from the streaked plate and inoculated into a flask containing 200 mL of sterile Tryptic Soy Broth (TSB). The flask was incubated at 35°C and rotated 150 rpm ± 10 rpm on an incubator shaker for 16-18 hr.

Procedure for Inoculation of the MBEC Plate:

The *P. aeruginosa* broth culture was diluted (approximately 1 x 10^8 CFU/mL) by pipetting 10 µL from the O/N flask, placing it in 100 mL of TSB to yield a cell density of 1 x 10^5 CFU/mL and mixing thoroughly. Both the O/N cultures and the 1 x 10^5 CFU/mL density cultures were enumerated using a serial-dilution spread plate technique.

A 150 µL aliquot of the *P. aeruginosa* culture (1 x 10^5 CFU/mL) was pipetted into columns 6, 7 and 8 [1:100 RelyOn™] and column 12 of a sterile MBEC plate. A 150 µL aliquot of sterile TSB was pipetted into column 10. The MBEC peg lid was carefully placed on the bottom of the Nunc plate to make sure there was no spillover from one well to another, incubated at 35°C, and shaken at 110 rpm ± 10 rpm on an incubator shaker for 16-18 hr.

Biofilm Growth Check:

The MBEC peg lid was rinsed two times for approximately 1 min each with 200 µL/well of Phosphate Buffered Saline (PBS) that had been pipetted into two separate 96-well Falcon round bottom 96-well tissue culture plates. Using sterile curved hemostats, four pegs were randomly selected and aseptically removed from column 12. Each peg was placed into 1 mL of phosphate buffered water (PBW). In a separate sterile Nunc 96 well plate a 200 µL aliquot of a 1:100 dilution of RelyOn™ was added to each well in columns 6, 7, and 8. TSB alone was pipetted into columns 10 and 12.

The biofilm was exposed to the 1:100 dilution of RelyOn™ for a 4-hr contact time. All pegs removed from the MBEC plate were sonicated for 30 min. The *P. aeruginosa* biofilm on the pegs was enumerated by a serial-dilution spread plating technique to determine the cell density (i.e., CFU/mL) after rinsing with PBS. At the end of each exposure time the MBEC lids were each placed onto a new 96-well Nunc plate containing 200 µL of D/E Neutralizing Broth (0.6% sodium thiosulfate) for 10 min to neutralize the RelyOn™.

The MBEC pegs/lid were rinsed by placing them into PBS (200 µL/well) twice in separate 96-well plates for 1 min each to remove any planktonic cells. Prior to sonicating, the MBEC pegs/lid were placed on the 96-well plate containing 200 µL/well of TBS to remove the biofilm from the pegs. The MBEC plate was then placed in an aluminum tray that floated in the sonicator water bath and sonicated at maximum power for 30 min to remove the biofilm. After sonication, the MBEC lid was discarded. The bottom portion of the MBEC plate (Recovery Plate) contained the biofilm that was quantitatively enumerated.

Enumeration of Exposure Wells

Four wells were randomly selected which were exposed to RelyOn™ and 100 µL of the content of the wells were placed into wells A1-12 in a new sterile 96 well plate. The wells chosen for the 1:100 dilution of RelyOn™ were C6, D6, E6, and F6 each containing 100 µL; the positive control wells each had 100 µL (B12, D12, F12 and H12). The remainder of the plate B1-H12 had 180 µL of PBW in the wells.

Serial dilutions were done in the 96-well plate by taking 20 µL from Row A and placing in Row B, mixing the contents (pipette tips changed) and pipetting 20 µL again from B to C. This pattern was followed throughout the remainder of the plate. These 1:10 dilutions resulted in dilutions of 10^{-0} thru 10^{-7}.

To enumerate any surviving cells, TSA plates were sectioned into quadrants and 10 µl of each dilution was plated into each quadrant. This scheme was followed for the rest of the microtiter plate.

Qualitative Measurement of Exposure Wells

From the Recovery Plate, 100 µL was pipetted into a new sterile 96-well plate followed by the addition of 100 µL of TSB to each well. The plate was placed in an incubator for 18-24 hr at 35°C.

Test Substance Preparation and Measurement of Activity (RelyOn™):

Samples	Trial	Ws	Vol thio 0.1	% $KHSO_5$	% AO
Bucket #1 Lot # 1502260974	1	0.4958	14.11	21.65	2.28
	2	0.5784	16.69	21.96	2.31
	3	0.4467	11.82	20.13	2.12
	4	0.4036	11.30	21.30	2.24
			Average	**21.26**	**2.24**
Bucket #2 Lot # 1502260975	1	0.4845	14.08	22.11	2.32
	2	0.5102	14.55	21.70	2.28
	3	0.5890	16.86	21.78	2.29
	4	0.4679	12.60	20.49	2.15
			Average	**21.52**	**2.26**

NOTE: The above chemical analyses were performed by Stephanie Tse, Associate Investigator, Chemours, Chemical Solutions, Clean & Disinfect

RESULTS:

The MBEC/ASTM technique for quantification/inhibition of biofilm is an excellent screening tool for determining the efficacy of a treatment(s) on viability of cells comprising a biofilm (i.e., the minimum inhibitory concentration, MIC) and the removal of the biofilm (i.e., minimum biofilm eradication concentration, MBEC). The study reported herein was a basic screen to see if 1% RelyOn™ was capable of killing *P. aeruginosa* in a biofilm and removing the 16-18-hr *P. aeruginosa* biofilm.

Two batches of RelyOn™ were examined for chemical activity (i.e., % active oxygen) and found to be within the acceptable range (see 'Test Substance Preparation and Measurement of Activity' section above). The 1:100 dilutions were then prepared according to label directions.

Table 1 shows the results from the determination of the inoculum planktonic cell density (8.4×10^4 CFU/mL) and the cell density of the 16 to18-hr biofilm from untreated biofilm peg removal (i.e., 2.04×10^5 CFU/mm^2). These quantitative measurements were found to be within the acceptable range (1.0×10^4 -1.0×10^6 CFU/mm^2) for a successful MBEC test.

The biofilm grown above was then subjected to treatment with a 1:100 dilution of RelyOn™ for 4 hr. During this period the positive control biofilm grew to a cell density of 5.15×10^5 CFU/mm^2 (see Table 2) indicating the viability of this *P. aeruginosa* biofilm during the exposure period. The RelyOn™ treated biofilm, on the other hand, only had a cell density of 2.14×10^0 CFU/mm^2, at the low level of detection for this method and indicating that there were no viable cells remaining after the 4-hr treatment (see Table 2). So the MIC for RelyOn™ against the *P. aeruginosa* biofilm cells was ≤ 1% and represented a 5.4 $\log_{10}$ reduction in cell density.

In addition to determining how effective the RelyOn™ was against the *P. aeruginosa* cells in the biofilm, the MBEC also determined the level of biofilm removal from the surface by monitoring absorbance readings at 650nm (A_{650}). The A_{650} results in Table 3 demonstrated that a 1:100 use dilution of RelyOn™ was effective in completely removing (i.e., 100%) the *P. aeruginosa* biofilm after a 4-hr contact time.

CONCLUSIONS:

RelyOn™ when used at a 1:100 dilution was very efficacious against *P. aeruginosa* cells in a 16 to 18-hr biofilm (≥ 5.4 $\log_{10}$ reduction) and demonstrated 100% removal of the biofilm after a 4-hr contact time in this MBEC assay.

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TABLE 1

Results from the enumeration of pulled pegs of untreated biofilm controls in an MBEC Assay.

Sample #	Organism - *P. aeruginosa* ATCC 15442	Expected Bioburden	Countable Dilution Factor	Volume Plated (µL)	Plate Counts	Bioburden (CFU/mL)	CFU/mm^2 *	Mean CFU/mm^2	± SD CFU/mm^2
NA	O/N Culture	1.00E+08	1.00E-05	100	128 368	2.48E+08	NA	NA	NA
NA	Diluted culture	1.00E+05	1.00E-02	100	81 87	8.40E+04	NA	NA	NA
A12	Untreated Biofilm of *P. aeruginosa* 4-hr MBEC plate	NA	1.00E-04	100	68 34	5.10E+06	1.09E+05	**2.04E+05**	1.50E+05
B12			1.00E-04	100	69 21	4.50E+06	9.65E+04		
E12			1.00E-05	100	20 23	2.15E+07	4.61E+05		
G12			1.00E-04	100	69 71	7.00E+06	1.50E+05		

*each peg surface area is 46.63 mm^2 O/N = Over Night SD = Standard Deviation CFU = Colony Forming Units

TABLE 2

Results from the spot plate counts of RelyOn™ treated wells at 0.5 hr.

Sample ID	RelyOn™ at 4 hr	Dilution Factor	Volume Plated (µL)	Plate Count	Bioburden (CFU/mL)	CFU/mm^2	Log_{10} Reduction
D10	Blank	1.00E+00	10	1	1.00E+02	2.14E-01	NA
B12	Untreated *P. aeruginosa* Biofilm	1.00E-04	10	6	6.00E+06	1.29E+05	NA
D12		1.00E-04	10	4	4.00E+07	8.58E+05	NA
F12		1.00E-05	10	3	3.00E+07	6.43E+05	NA
H12		1.00E-05	10	2	2.00E+07	4.29E+05	NA
				MEAN	**2.40E+07**	**5.15E+05**	NA
				SD	**1.26E+07**	**2.70E+05**	NA
C4	1:100 RelyOn™	1.00E+00	10	1	1.00E+02	2.14E+00	5.4
D4		1.00E+00	10	1	1.00E+02	2.14E+00	5.4
E4		1.00E+00	10	1	1.00E+02	2.14E+00	5.4
F4		1.00E+00	10	1	1.00E+02	2.14E+00	5.4
						MEAN	**5.4**
						SD	**0.0**

TABLE 3

Gross and Net A_{650} values for the 16-18 hr *P. aeruginosa* biofilms exposed to RelyOn™ at a 1:100 use dilution for 4 hr.

Row	1:100 RelyOn™			TSB Control	Biofilm Control
A	0.044	0.045	0.054	0.051	
B	0.046	0.047	0.048		2.279
C	0.044	0.046	0.045	0.051	
D	0.046	0.048	0.050		2.322
E	0.048	0.049	0.050	0.049	
F	0.050	0.049	0.048		2.277
G	0.043	0.042	0.042	0.043	
H	0.052	0.048	CONTAM	0.048	2.247
Gross Mean A_{650}	0.047			0.048	2.281
± SD	0.003			0.004	0.031
CV (%)	6.5			7.8	1.4

Row	1:100 RelyOn™			TSB Control	Biofilm Control
A	-0.004	-0.003	0.006	0.003	
B	-0.002	-0.001	0.000		2.231
C	-0.004	-0.002	-0.003	0.003	
D	-0.002	0.000	0.002		2.274
E	0.000	0.001	0.002	0.001	
F	0.002	0.001	0.000		2.229
G	-0.005	-0.006	-0.006	-0.005	
H	0.004	0.000	CONTAM	0.000	2.199
Net A_{650} Mean	**-0.001**			**0.000**	**2.233**
± SD	0.003			0.003	0.031
CV (%)	0.0			0.0	1.4
% Reduction	**100.06**				
LEGEND					
Spot Plate Enumerated		Denotes Peg Removal		2.04E+05 ± 1.50E+05 CFU/mm^2	