



The Efficacy of Cleancert® against bacterial biofilms

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1. Background

Biofilms are complex communities of microorganisms that reside in a self-produced matrix. This matrix is referred to as extracellular polymeric substances (EPS). Biofilms are a major health concern, particularly in the context of healthcare-associated infections, due to their increased recalcitrance to a wide range of antimicrobials. Biofilms are associated with persistent infections such as periodontitis, chronic wounds and septicaemia to name but a few. Therefore the development of technologies that can effectively eradicate the biofilm are of great importance.

2. Research Aims

To test the effectiveness of Cleancert® on *Pseudomonas aeruginosa* NCTC 10662 and *Staphylococcus aureus* ATCC 25923 biofilms against Orotol using the MBEC assay.
To test the effectiveness of Cleancert on *Pseudomonas aeruginosa* NCTC 10662 and *Staphylococcus aureus* ATCC 25923 24- and 72-hour biofilms against Orotol using the CDC biofilm reactor.

2. Experimental Procedure for Minimum Biofilm Eradication Concentration (MBEC) Assay

The experimental procedure for the MBEC assay was adapted from ASTM E2799-12 Standard Test Method for Testing Disinfectant Efficacy against *Pseudomonas aeruginosa* biofilm using the MBEC Assay.

3.1 Preparation of the biofilms

Tryptone soya broth (TSB) (Oxoid, Hampshire, UK) was inoculated with bacteria to a concentration of 10^8 CFU/ml, which was determined by diluting cultures to an optical density of 0.19 using a spectrometer (equivalent to McFarland standard 0.5, approximately 10^8 CFU/ml). The 10^8 CFU/ml culture was diluted to 10^7 CFU/ml before adding 150µl of the suspension to the wells of the microtitre plate. The same volume of TSB was used as a sterility control. The microtitre plate was covered with a 96-peg lid and incubated in a humidified incubator for 24 hours at 37°C (+/- 2C), with gentle shaking at 40rpm.

The 24-hour biofilms on the peg lid were placed in 0.85% saline solution for 2 minutes to remove planktonic bacteria. The biofilms were then transferred to the challenge plate and incubated for 24 hours at 37°C. For TSB sterility controls, several wells of the microtitre plate were not inoculated with bacteria and therefore the wells contained TSB alone. The biofilms were removed from the challenge plate and washed in 0.85% saline solution.

3.2 Preparation of the challenge plate

Dilutions of all antimicrobials were prepared using TSB except for neat solutions. Stock solutions of each substance were filter sterilised (0.2µm filter) and serially diluted in a 96-well plate. For the sterility control specific wells of the plate contained TSB without antimicrobial. Similarly, no antimicrobial was prepared for specific wells containing bacteria for a positive growth control (see Figure 1).

3.3 Determination of minimum inhibitory concentration (MIC)

To determine the MIC of the antimicrobials, an initial absorbance reading of all wells was performed before the resulting challenge plate was incubated for 24 hours at 37°. Following incubation, the turbidity of the wells was observed and the absorbance of each well of the microtitre plate was measured at 650nm. Wells that indicated no bacterial growth had absorbance readings of <0.1. To ensure that this figure was accurate for no growth, the absorbance readings before incubation and after incubation were compared. An increase in absorbance indicated that there was bacterial growth. For the purpose of this report, absorbance values taken post-incubation have been included. The MIC is defined as the minimum concentration of antimicrobial that inhibits growth of the dispersed cells from the biofilm. Clear wells are evidence of inhibition following a suitable period of incubation.

3.4 Determination of minimum bactericidal concentration (MBC)

To determine the MBC, a 20µl sample from each well of the challenge plate was added to 180µl antimicrobial neutraliser (see Appendix 1) and TSB. The plate was incubated for 24 hours and turbidity observed and measured at 650nm, whereby no bacterial growth has absorbance readings of <0.1. The MBC value represents the lowest concentration of antimicrobial, which kills 99.9% of the population of the dispersed cells that have been shed from the biofilm. Clear wells are evidence of effective biocidal concentration following a suitable period of incubation.

3.5 Determination of minimum biofilm eradication concentration (MBEC)

To determine the MBEC of antimicrobials, treated biofilms were then transferred to a new microtitre plate of antimicrobial neutraliser (see Appendix 1) and TSB (90µl /well). Biofilms were removed from the pegs and into the neutraliser/TSB solution using sonication for 30 minutes. The peg lid was finally removed and discarded. Sonicated biofilms in neutraliser/TSB were incubated for 24 hours at 37°C. Turbidity of the wells was observed and the absorbance was measured at 650nm, whereby no bacterial growth has absorbance readings of <0.1. The MBEC is defined as the

minimum concentration of antimicrobial that eradicates the biofilm. Clear wells were evidence of eradication following a suitable period of incubation.

4. Results

Treatment of *P. aeruginosa* 24-hour biofilms with Cleancert for various treatment times resulted in MIC, MBC and MBEC values of 100% (neat concentration), whereas *S. aureus* biofilms treated for 1 and 30 minutes resulted in MIC values of 50% and 25% for 1 hour treatment. MBC and MBEC values for *S. aureus* biofilms treated with Cleancert showed values of 100% (neat concentration).

The MIC values for Orotol Plus were higher than Orotol MD555 for both *S. aureus* and *P. aeruginosa* biofilms, however the MIC values for both Orotol Plus and Orotol MD555 were lower than those for Cleancert. For the shorter treatment times (1 minute, 30 minutes and 1 hour), Asepticcate (8%) showed equivalent MIC values to Orotol MD555 for *S. aureus* biofilms (6.25%). The treatment of *P. aeruginosa* biofilms with Asepticcate (8%) resulted in variable MIC values.

The MBC values for the treatment of *S. aureus* and *P. aeruginosa* biofilms with Orotol Plus could not be obtained for the shorter treatment times, as growth was detected in samples treated with neat concentrations. However, an MBC value of 6.25% and 12.5%, respectively, was obtained for 24-hour treatment. Low MBC values ranging between 6.25%-12.5% were observed for all biofilms treated with Orotol MD555, whereas slighter higher values were determined for Asepticcate (8%) (6.25%-50%).

None of the tested antimicrobials showed *P. aeruginosa* biofilm eradication after 1 minute treatment time, however Cleancert and Orotol Plus eradicated *S. aureus* biofilm after 1 minute at 100% and 6.25% respectively. Cleancert then showed biofilm eradication at 30 minutes, 1 hour and 24 hours treatment of all biofilms, with MBEC values of 100%. Orotol Plus showed biofilm eradication at various concentrations, with MBEC values ranging from 6.25% to 100%. Orotol MD555 displayed varying MBEC values for all biofilms treated for 30 minutes, 1 hour and 24 hours from 6.25% to 100% and in some cases, no eradication was determined. Asepticcate (8%) did not eradicate biofilms at 30 minutes but MBEC values ranging between 6.25% and 25% for 1 hour and 24 hour treatment were recorded.

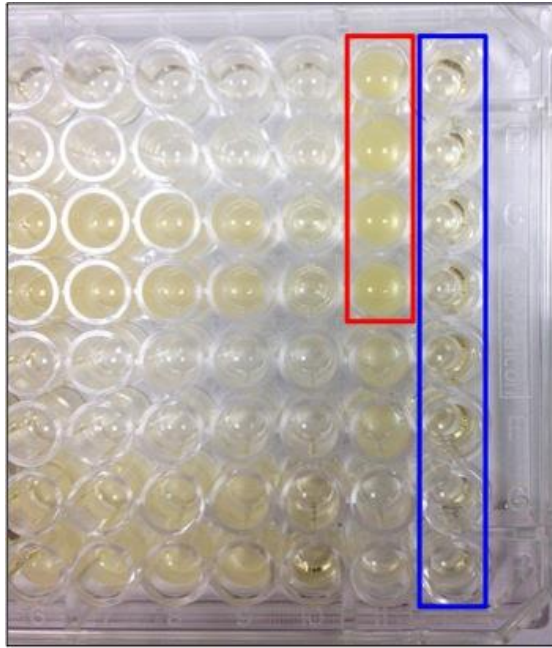


Figure 1. 96-well plate in the MBEC assay. The red line indicates wells containing bacterial without antimicrobial (positive growth control) and the blue line indicates wells containing only TSB and no antimicrobial or bacteria (negative, no growth control).

	<i>P. aeruginosa</i>					<i>S. aureus</i>				
	Percentage Antimicrobial* (%)					Percentage Antimicrobial* (%)				
Antimicrobial	6.25	12.5	25	50	100	6.25	12.5	25	50	100
Untreated control	1.63425					0.602				
Cleancert	1.272	1.163	1.5785	1.1535	-0.001	0.4215	0.901	0.308	0.0005	0.0015
Orotol Plus	0.538	0.2025	0.058	0.0375	0.0015	0.525	0.1305	0.006	0.003	-0.0005
Orotol MD555	0.041	0.0155	0.013	0.006	0.002	0.0105	0.0085	0.0055	0.0035	0.0145
Asepticcate (8%)	0.338	0.132	0.008	0.0035	0.003	0.0035	0.0035	0.002	0.003	0.003

Table 1A. Average absorbance values (650nm) of the MIC plate following antimicrobial treatment for 1 minute. *Percentage antimicrobial indicates the percentage of stock solutions. Stock solutions were as follows: Cleancert: neat; Orotol Plus: neat; Orotol MD555: neat, and Asepticcate (Tetrasodium EDTA): 8%. Grey shading indicates growth, where the absorbance values are >0.1. A value less than an OD of 0.1 was interpreted an indication of no growth.

	<i>P. aeruginosa</i>					<i>S. aureus</i>				
	Percentage Antimicrobial* (%)					Percentage Antimicrobial* (%)				
Antimicrobial	6.25	12.5	25	50	100	6.25	12.5	25	50	100
Untreated control	2.22025					0.72575				
Cleancert	1.844	1.839	1.9345	1.9895	0.0005	0.638	0.976	0.8285	0.719	0.015
Orotol Plus	0.053	0.141	0.689	0.9115	0.353	0.066	0.3425	0.6475	0.823	0.3125
Orotol MD555	0.01	0.01	0.0105	0.0095	0.013	0.019	0.0235	0.025	0.0215	0.02
Asepticcate (8%)	1.238	0.613	0.3965	0.001	-0.009	0.637	0.006	0.0045	0.008	0.0135

Table 1B. Average absorbance values (650nm) of the MBC plate following antimicrobial treatment for 1 minute. *Percentage antimicrobial indicates the percentage of stock solutions. Stock solutions were as follows: Cleancert: neat; Orotol Plus: neat; Orotol MD555: neat, and Asepticcate (Tetrasodium EDTA): 8%. Grey shading indicates growth, where the absorbance values are >0.1. A value less than an OD of 0.1 was interpreted an indication of no growth.

	<i>P. aeruginosa</i>					<i>S. aureus</i>				
	Percentage Antimicrobial* (%)					Percentage Antimicrobial* (%)				
Antimicrobial	6.25	12.5	25	50	100	6.25	12.5	25	50	100
Untreated control	2.242					0.646				
Cleancert	1.29275	2.03525	2.06625	1.57575	0.57475	1.25025	0.51425	0.45725	0.44025	0.03175
Orotol Plus	0.07625	0.09825	0.44175	0.06525	0.33525	0.01625	0.01975	0.01875	0.01675	0.01425
Orotol MD555	0.29375	0.75525	0.34275	0.33875	0.59425	0.21025	0.46225	0.65675	0.39425	0.69525
Asepticite (8%)	0.09975	0.09275	0.15275	0.07125	0.10675	0.19975	0.35625	0.20975	0.19825	0.14575

Table 1C. Average absorbance values (650nm) of the MBEC plate following antimicrobial treatment for 1 minute. *Percentage antimicrobial indicates the percentage of stock solutions. Stock solutions were as follows: Cleancert: neat; Orotol Plus: neat; Orotol MD555: neat, and Asepticite (Tetrasodium EDTA): 8%. Grey shading indicates growth, where the absorbance values are >0.1. A value less than an OD of 0.1 was interpreted an indication of no growth.

Antimicrobial	<i>P. aeruginosa</i>					<i>S. aureus</i>				
	Percentage Antimicrobial* (%)					Percentage Antimicrobial* (%)				
	6.25	12.5	25	50	100	6.25	12.5	25	50	100
Untreated control	1.648					0.36925				
Cleancert	1.41325	1.36775	1.33925	1.31625	-0.03025	0.45475	0.39825	0.33325	-0.02925	-0.03075
Orotol Plus	0.47725	0.11725	0.02075	0.01325	-0.02725	0.52025	0.12275	-0.01975	-0.02825	-0.02875
Orotol MD555	-0.00025	-0.01175	-0.01825	-0.02175	-0.02625	-0.02425	-0.02125	-0.02375	-0.02375	-0.02525
Asepticcate (8%)	0.26125	0.01175	-0.01825	-0.02375	-0.02575	-0.02375	-0.02475	-0.02425	-0.02525	-0.02575

Table 2A. Average absorbance values (650nm) of the MIC plate following antimicrobial treatment for 30 minutes. *Percentage antimicrobial indicates the percentage of stock solutions. Stock solutions were as follows: Cleancert: neat; Orotol Plus: neat; Orotol MD555: neat, and Asepticcate (Tetrasodium EDTA): 8%. Grey shading indicates growth, where the absorbance values are >0.1. A value less than an OD of 0.1 was interpreted an indication of no growth.

	<i>P. aeruginosa</i>					<i>S. aureus</i>				
	Percentage Antimicrobial* (%)					Percentage Antimicrobial* (%)				
Antimicrobial	6.25	12.5	25	50	100	6.25	12.5	25	50	100
Untreated control	2.20525					0.514				
Cleancert	2.07225	2.31225	2.16225	1.98575	0.00175	0.69675	0.64975	0.67575	0.61525	0.01825
Orotol Plus	0.05725	0.16225	0.72175	0.94325	0.26925	0.07225	0.26075	0.67475	0.95375	0.26475
Orotol MD555	0.00925	0.00775	0.01125	0.01025	0.00825	0.02475	0.02375	0.02525	0.02425	0.02075
Asepticcate (8%)	1.22925	0.56575	0.30225	-0.00575	-0.00925	0.76675	0.00525	0.00825	0.00925	0.01475

Table 2B. Average absorbance values (650nm) of the MBC plate following antimicrobial treatment for 30 minutes. *Percentage antimicrobial indicates the percentage of stock solutions. Stock solutions were as follows: Cleancert: neat; Orotol Plus: neat; Orotol MD555: neat, and Asepticcate (Tetrasodium EDTA): 8%. Grey shading indicates growth, where the absorbance values are >0.1. A value less than an OD of 0.1 was interpreted an indication of no growth.

	<i>P. aeruginosa</i>					<i>S. aureus</i>				
	Percentage Antimicrobial* (%)					Percentage Antimicrobial* (%)				
Antimicrobial	6.25	12.5	25	50	100	6.25	12.5	25	50	100
Untreated control	2.039					0.821				
Cleancert	2.13425	1.80425	1.26825	1.15575	0.02375	0.89975	0.63625	0.45775	0.38025	0.00725
Orotol Plus	-0.00675	-0.00075	0.01375	-0.00275	-0.00325	0.00075	0.00375	0.00175	0.00175	0.00075
Orotol MD555	0.63575	0.02775	0.02525	0.02325	0.04475	0.84175	0.12925	0.21825	0.22475	0.91225
Asepticcate (8%)	0.15925	0.05125	0.11925	0.13025	0.11275	0.69375	0.22725	0.16975	0.47825	0.60825

Table 2C. Average absorbance values (650nm) of the MBEC plate following antimicrobial treatment for 30 minutes. *Percentage antimicrobial indicates the percentage of stock solutions. Stock solutions were as follows: Cleancert: neat; Orotol Plus: neat; Orotol MD555: neat, and Asepticcate (Tetrasodium EDTA): 8%. Grey shading indicates growth, where the absorbance values are >0.1. A value less than an OD of 0.1 was interpreted an indication of no growth.

	<i>P. aeruginosa</i>					<i>S. aureus</i>				
	Percentage Antimicrobial* (%)					Percentage Antimicrobial* (%)				
Antimicrobial	6.25	12.5	25	50	100	6.25	12.5	25	50	100
Untreated control	1.78575					0.756				
Cleancert	1.4705	1.8165	2.148	0.5825	-0.001	0.617	0.1395	0.008	-0.001	-0.003
Orotol Plus	0.5665	0.127	0.009	0.001	-0.0035	0.5355	0.1395	0.008	-0.001	-0.003
Orotol MD555	0.0245	0.006	0.0025	0.0035	0.0005	0.004	0.0025	0.001	0.002	0.0025
Asepticcate (8%)	0.002	-0.0015	-0.002	-0.0035	-0.0035	-0.001	0.0015	0.0025	-0.0015	0.0045

Table 3A. Average absorbance values (650nm) of the MIC plate following antimicrobial treatment for 1 hour. *Percentage antimicrobial indicates the percentage of stock solutions. Stock solutions were as follows: Cleancert: neat; Orotol Plus: neat; Orotol MD555: neat, and Asepticcate (Tetrasodium EDTA): 8%. Grey shading indicates growth, where the absorbance values are >0.1. A value less than an OD of 0.1 was interpreted an indication of no growth.

	<i>P. aeruginosa</i>					<i>S. aureus</i>				
	Percentage Antimicrobial* (%)					Percentage Antimicrobial* (%)				
Antimicrobial	6.25	12.5	25	50	100	6.25	12.5	25	50	100
Untreated control	1.9495					0.681				
Cleancert	2.253	2.317	2.14	1.876	-0.0145	0.6605	0.668	0.6185	0.535	-0.013
Orotol Plus	0.0475	0.232	0.799	0.9305	0.31	0.0495	0.227	0.7365	0.8465	0.268
Orotol MD555	-0.0095	-0.0115	-0.0075	-0.002	-0.0065	-0.01	-0.006	-0.005	-0.0075	-0.0065
Asepticcate (8%)	1.042	0.195	-0.0145	-0.0145	-0.0155	0.621	-0.0175	-0.0185	-0.017	-0.014

Table 3B. Average absorbance values (650nm) of the MBC plate following antimicrobial treatment for 1 hour. *Percentage antimicrobial indicates the percentage of stock solutions. Stock solutions were as follows: Cleancert: neat; Orotol Plus: neat; Orotol MD555: neat, and Asepticcate (Tetrasodium EDTA): 8%. Grey shading indicates growth, where the absorbance values are >0.1. A value less than an OD of 0.1 was interpreted an indication of no growth.

	<i>P. aeruginosa</i>					<i>S. aureus</i>				
	Percentage Antimicrobial* (%)					Percentage Antimicrobial* (%)				
Antimicrobial	6.25	12.5	25	50	100	6.25	12.5	25	50	100
Untreated control	1.77675					0.877				
Cleancert	1.0555	1.045	0.9425	0.751	-0.0035	1.2935	1.3265	0.6705	0.3095	-0.0015
Orotol Plus	0.1085	0.035	0.1035	0.005	0.0035	1.086	0.019	0.669	1.0445	0.0115
Orotol MD555	0.1925	0.01	0.063	0.031	0.1175	0.4675	1.0635	0.983	2.017	0.0965
Asepticcate (8%)	0.077	0.084	0.0495	0.033	0.0265	0.1155	0.1105	0.085	0.0745	0.086

Table 3C. Average absorbance values (650nm) of the MBEC plate following antimicrobial treatment for 1 hour. *Percentage antimicrobial indicates the percentage of stock solutions. Stock solutions were as follows: Cleancert: neat; Orotol Plus: neat; Orotol MD555: neat, and Asepticcate (Tetrasodium EDTA): 8%. Grey shading indicates growth, where the absorbance values are >0.1. A value less than an OD of 0.1 was interpreted an indication of no growth.

	<i>P. aeruginosa</i>					<i>S. aureus</i>				
	Percentage Antimicrobial* (%)					Percentage Antimicrobial* (%)				
Antimicrobial	6.25	12.5	25	50	100	6.25	12.5	25	50	100
Untreated control	1.4595					1.488				
Cleancert	1.35	1.3255	1.35	1.327	0.016	0.7675	1.0155	0.854	0.386	0.025
Orotol Plus	0.403	0.6635	0.2	0.0495	-0.06	0.7305	0.6425	0.303	0.07	0.036
Orotol MD555	-0.2715	-0.191	-0.1575	-0.2	-0.32	-0.3085	-0.3145	-0.2695	-0.268	-0.3795
Asepticcate (8%)	0.0985	0.0455	0.023	-0.005	-0.007	0.1005	0.074	0.0835	0.0695	0.0725

Table 4A. Average absorbance values (650nm) of the MIC plate following antimicrobial treatment for 24 hours. *Percentage antimicrobial indicates the percentage of stock solutions. Stock solutions were as follows: Cleancert: neat; Orotol Plus: neat; Orotol MD555: neat, and Asepticcate (Tetrasodium EDTA): 8%. Grey shading indicates growth, where the absorbance values are >0.1. A value less than an OD of 0.1 was interpreted an indication of no growth.

	<i>P. aeruginosa</i>					<i>S. aureus</i>				
	Percentage Antimicrobial* (%)					Percentage Antimicrobial* (%)				
Antimicrobial	6.25	12.5	25	50	100	6.25	12.5	25	50	100
Untreated control	1.68375					0.766				
Cleancert	1.5355	2.119	1.9195	2.035	0.075	1.222	1.3645	1.1285	1.502	-0.1095
Orotol Plus	-0.4715	-0.0455	-0.148	-0.0825	0.046	0.683	-0.1355	-0.134	-0.098	0.038
Orotol MD555	0.2095	-0.136	-0.222	0.0325	0.5935	-0.2775	-0.2495	-0.2435	0.013	0.558
Asepticcate (8%)	0.0575	0.01	0.0005	0.0145	0.253	-0.009	-0.0645	0.124	-0.0075	0.0455

Table 4B. Average absorbance values (650nm) of the MBC plate following antimicrobial treatment for 24 hours. *Percentage antimicrobial indicates the percentage of stock solutions. Stock solutions were as follows: Cleancert: neat; Orotol Plus: neat; Orotol MD555: neat, and Asepticcate (Tetrasodium EDTA): 8%. Grey shading indicates growth, where the absorbance values are >0.1. A value less than an OD of 0.1 was interpreted an indication of no growth.

	<i>P. aeruginosa</i>					<i>S. aureus</i>				
	Percentage Antimicrobial* (%)					Percentage Antimicrobial* (%)				
Antimicrobial	6.25	12.5	25	50	100	6.25	12.5	25	50	100
Untreated control	1.81575					0.78975				
Cleancert	1.887	1.4715	1.412	1.6725	0.1035	1.2515	1.2485	1.2065	1.33	-0.0885
Orotol Plus	-0.1975	-0.112	-0.1025	-0.103	-0.1135	-0.1055	-0.0215	-0.0305	-0.016	0.007
Orotol MD555	-0.1335	-0.048	-0.03	-0.0375	0.007	-0.1775	-0.123	-0.084	-0.065	-0.096
Asepticcate (8%)	-0.177	-0.186	-0.1925	0.0725	0.3115	-0.2025	-0.1975	-0.174	-0.2	-0.0775

Table 4C. Average absorbance values (650nm) of the MBEC plate following antimicrobial treatment for 24 hours. *Percentage antimicrobial indicates the percentage of stock solutions. Stock solutions were as follows: Cleancert: neat; Orotol Plus: neat; Orotol MD555: neat, and Asepticcate (Tetrasodium EDTA): 8%. Grey shading indicates growth, where the absorbance values are >0.1. A value less than an OD of 0.1 was interpreted an indication of no growth.

Antimicrobial	<i>P. aeruginosa</i>											
	MIC				MBC				MBEC			
	1 min	30 min	1h	24h	1 min	30 min	1h	24h	1 min	30 min	1h	24h
Cleancert	100	100	100	100	100	100	100	100	x	100	100	100
Orotol Plus	25	25	25	50	x	x	x	6.25	x	6.25	12.5	6.25
Orotol MD555	6.25	6.25	6.25	6.25	6.25	6.25	6.25	12.5	x	12.5	x	6.25
Asepticcate (8%)	25	12.5	25	6.25	50	50	25	6.25	x	x	6.25	6.25

Table 5A. Summary of MIC, MBC and MBEC values for *P. aeruginosa*. Values represent a percentage of the stock antimicrobial solution. The 'x' denotes that all concentrations of the antimicrobial tested, including the antimicrobial in neat form, were ineffective.

Antimicrobial	<i>S. aureus</i>											
	MIC				MBC				MBEC			
	1 min	30 min	1h	24h	1 min	30 min	1h	24h	1 min	30 min	1h	24h
Cleancert	50	50	25	100	100	100	100	100	100	100	100	100
Orotol Plus	25	25	25	50	x	x	x	12.5	6.25	6.25	100	6.25
Orotol MD555	6.25	6.25	6.25	6.25	6.25	6.25	6.25	6.25	x	x	100	6.25
Asepticcate (8%)	6.25	6.25	6.25	6.25	12.5	12.5	12.5	6.25	x	x	25	6.25

Table 5B. Summary of MIC, MBC and MBEC values for *S. aureus*. Values represent a percentage of the stock antimicrobial solution. The 'x' denotes that all concentrations of the antimicrobial tested, including the antimicrobial in neat form, were ineffective.

5. Summary of results 1

- Cleancert (in neat form) inhibited the growth of dispersed planktonic cells from the biofilm, showed biocidal effects on dispersed planktonic cells and eradicated 24-hour biofilm. Diluted Cleancert was not effective against planktonic bacteria or those found within a biofilm.
- Orotol Plus (Neat and ranging from 6.25-50% concentration) also inhibited and had biocidal effects toward dispersed, planktonic bacteria and eradicated biofilm.
- Orotol MD555 (Neat and ranging from 6.25% and 12.5%) was also effective against dispersed planktonic cells and biofilm.
- Asepticate was effective on dispersed planktonic cells and biofilm at concentrations as low as 0.5%.
- Due to the variability of MIC, MBC and MBEC values at the shorter incubation times, it is recommended that the MBEC model is more suitable for 24-hour treatment periods.

6. Experimental Procedure for CDC Biofilm Reactor

The experimental procedure for the CDC biofilm reactor studies was adapted from ASTM E2871-13 Standard Test Method for Evaluating Disinfectant Efficacy Against *Pseudomonas aeruginosa* Biofilm Grown in a CDC Biofilm Reactor (see figure).

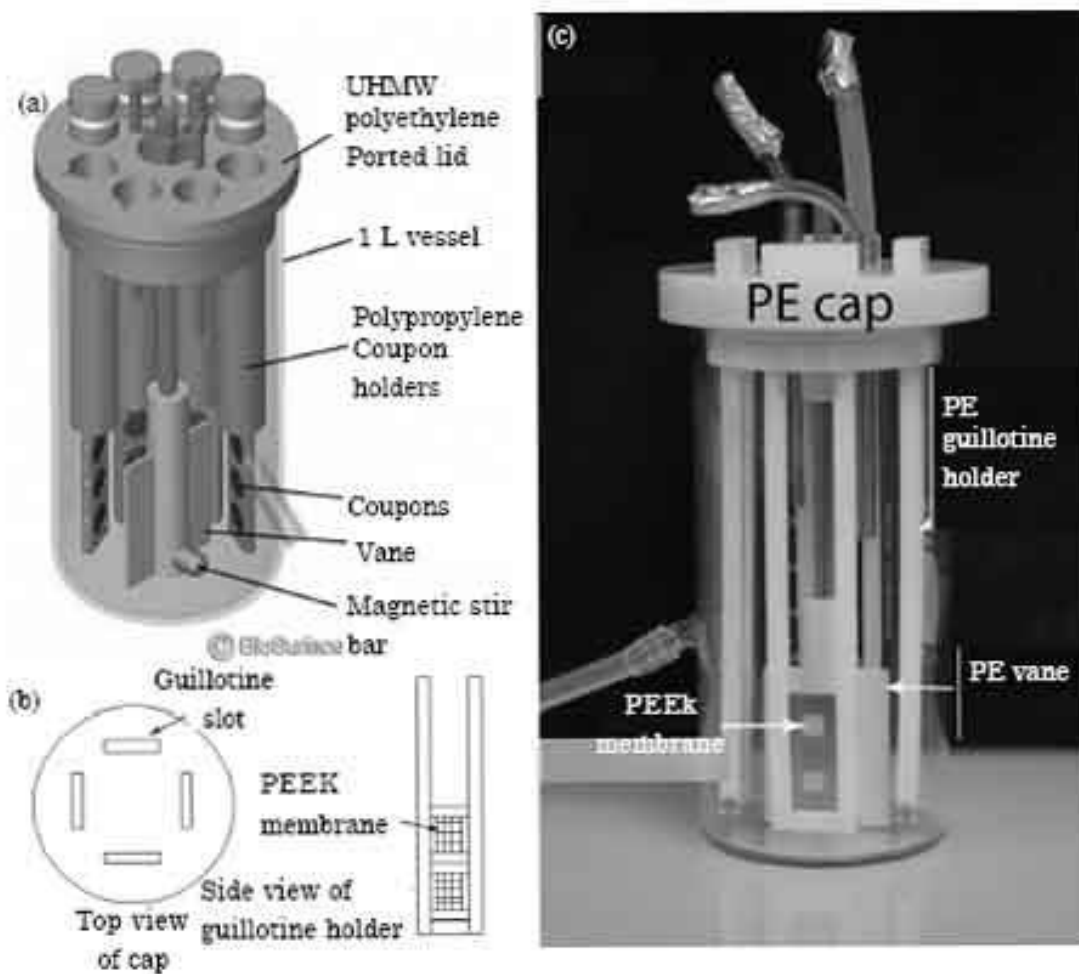


Figure. CDC Bioreactor

6.1 Culture/Inoculum preparation

Tryptone soya broth (TSB) (Oxoid, Hampshire, UK) was inoculated with bacteria to a concentration of 10^8 CFU/ml, which was determined by diluting cultures to an optical density of 0.19 using a spectrometer (equivalent to McFarland standard 0.5).

6.2 Biofilm formation

Each CDC reactor contains 8 polypropylene rods designed to hold three coupons. In this experiment, polycarbonate coupons were used. The CDC reactor was sterilised before aseptically adding 300ml of sterile TSB through the inoculation port. Following this, 1ml of the previously prepared 10^8 CFU/ml inoculum was then added to the reactor. The reactor was placed on a magnetic stir plate. The rotation speed was set to 125 ± 5 rpm. The CDC reactor was operated in batch mode at room temperature ($21 \pm 2^\circ\text{C}$) for 24 hours (24-hour biofilms) or 72 hours (72-hour biofilm).

6.3 Biofilm treatment

Following incubation, the rods containing the polycarbonate coupons were removed and rinsed in sterile 0.85% sodium chloride solution to remove planktonic cells. Each coupon was released from the rods into individual sterile 50ml centrifuge tubes. Each coupon was then treated with 3ml of sterile antimicrobial. Treatments of the coupons were performed in duplicate. Antimicrobials used included Cleancert (neat), Orotol MD555 (neat), Orotol Plus (neat) and Asepticate. The coupons were then incubated at 37°C for 1, 5 or 15 minute contact times. Following incubation, the antimicrobial was neutralised in 10ml of appropriate sterile neutraliser (see Appendix 1) and 10ml TSB. Each 50ml centrifuge tube containing the coupon and neutraliser was mixed using a vortex for 30 seconds, followed by sonication for 15 minutes. The disaggregated biofilm samples were sampled and serially diluted for bacterial enumeration using the spot plate technique, which was performed in triplicate (see Figure 2). Biofilm density was calculated Log₁₀ density for each coupon. The Log₁₀ density of each coupon was subtracted from the Log₁₀ density of the untreated control coupon to determine the Log₁₀ reduction value of each treated biofilm. Percentage kill was then calculated using the following formula:

$$\% \text{ kill} = (1 - 10^{-\text{Log reduction value}}) \times 100$$

Data for the Log₁₀ reduction and percentage kill (%) was presented in table form (see **Table 6A** and **6B**).

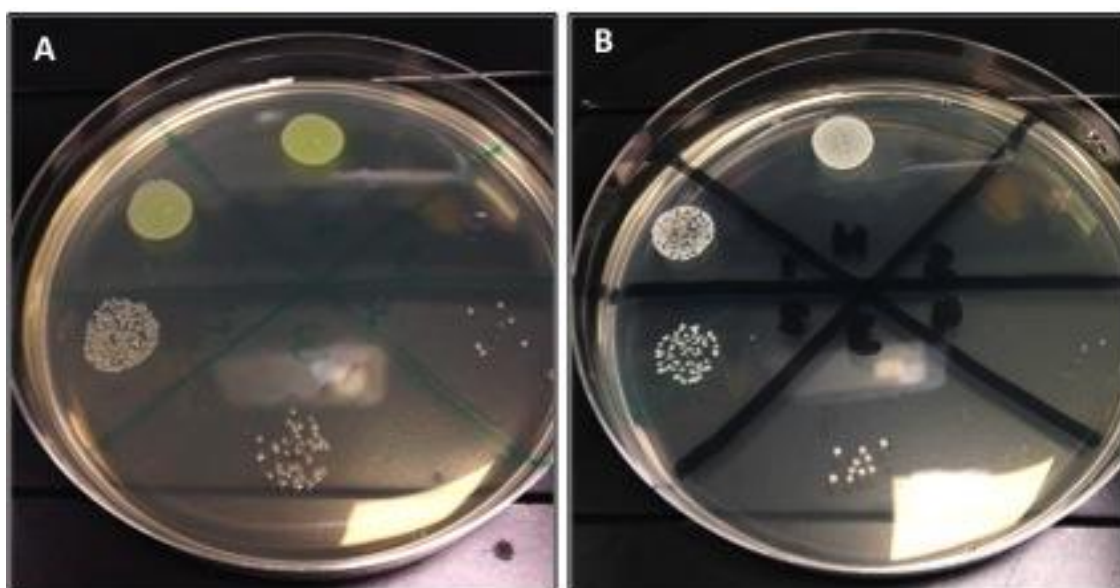


Figure 2. *P. aeruginosa* (A) and *S. aureus* (B) spot plate technique for the enumeration of bacterial suspension. Both images represent colonies obtained from serial dilutions of bacteria from an untreated 24-hour biofilm CDC coupon.

7. Results 2

The use of the CDC biofilm reactor was used to assess whether the antimicrobials are effective at short-contact times. The treatment of the CDC reactor *S. aureus* and *P. aeruginosa* 24-hour and 72-hour biofilms with a neat concentration of Orotol Plus an Orotol MD555 resulted in the detection of no bacterial colonies at all time points (see Table 6A and 6B). Given that the Log_{10} of zero is undefined, a number of 0.5 (colonies) was used to obtain a Log_{10} reduction value of 5.07 and a percentage kill of 99.99%. The treatment of *P. aeruginosa* 24-hour and 72-hour biofilms with Cleancert (neat) for 15 minutes also resulted in a Log_{10} reduction of 5.07 and a percentage kill of 99.99%. Similarly 72-hour *S. aureus* biofilms treated with Cleancert for 15 minutes showed a Log_{10} reduction value of 5.07 and a percentage kill of 99.99%. A Log_{10} reduction value of 3.33 ± 0.09 and percentage kill of 99.95% was calculated for 24-hour biofilms treated with Cleancert for 15 minutes.

Asepticase (4%) did not fully kill *S. aureus* and *P. aeruginosa* biofilms, however percentage kill values included 80.51% and 80.25% for *S. aureus* 24-hour and 72-hour biofilms respectively, and 99.51% and 95.07% for *P. aeruginosa* 24-hour and 72 hour biofilms respectively (see Figure 6A and 6B).

7.1 Observations

Upon treatment of the CDC bioreactor coupon with a neat concentration of Orotol MD555, the antimicrobial was neutralised and after a short incubation period at room temperature, a white substance was observed (see Figure 2). It is suspected that the substance was either due to the formation of a precipitate or the

breakdown of the polycarbonate coupons. The occurrence of the white substance was in all samples treated with Orotol MD555, however this was not observed for the other antimicrobials.

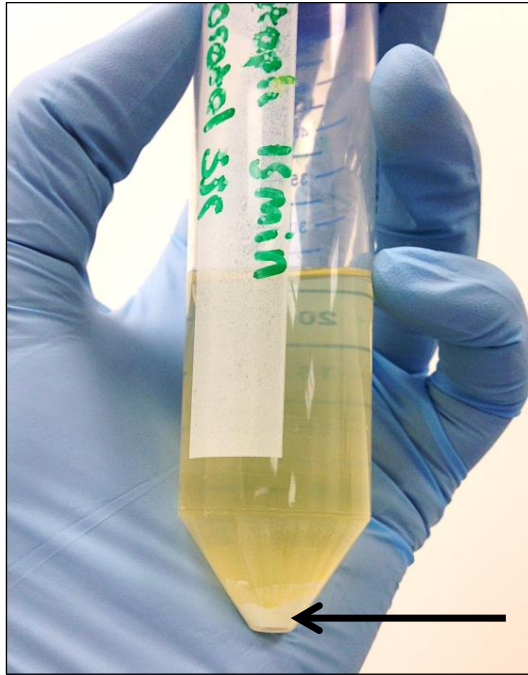


Figure 3. CDC bioreactor coupon in Orotol MD555 and neutraliser. The black arrow highlights the formation of a white substance following Orotol MD555 (neat) treatment and neutralisation.

	<i>S. aureus</i>											
	24-Hour Biofilms						72-Hour Biofilms					
Treatment Time	1		5		15		1		5		15	
Antimicrobial	LR	Kill (%)	LR	Kill (%)	LR	Kill (%)	LR	Kill (%)	LR	Kill (%)	LR	Kill (%)
Cleancert	3.09 ± 0.25	99.91	3.20 ± 0.26	99.94	3.33 ± 0.09	99.95	2.0 ± 0.21	98.99	3.37 ± 0.35	99.96	5.07	99.99
Orotol MD555	5.07	99.99	5.07	99.99	5.07	99.99	5.07	99.99	5.07	99.99	5.07	99.99
Orotol Plus	5.07	99.99	5.07	99.99	5.07	99.99	5.07	99.99	5.07	99.99	5.07	99.99
Asepticcate (4%)	1.59 ± 1.56	97.41	0.40 ± 0.05	59.90	0.71 ± 0.11	80.51	0.34 ± 0.37	54.09	0.70 ± 0.05	79.86	0.70 ± 0.02	80.25
Untreated control	Mean Log ₁₀ density of 6.93 ± 0.05 LR - 0 Kill (%) - 0						Mean Log ₁₀ density of 7.58 ± 0.05 LR - 0 Kill (%) - 0					

Table 6A. Log reduction (LR) and percentage kill (%) values of *S. aureus* biofilms.

Biofilms were cultured for 24 or 72 hours in a CDC biofilm reactor before treatment with Cleancert (neat), Orotol MD555 (neat), Orotol Plus (neat) or Asepticcate (Neat 4%) for 1, 5 or 15 minutes. Samples were performed in duplicate. Mean Log values were calculated and the standard error (SE) of the mean LR was determined (LR ± SE). Given that the log of zero is mathematically undefined, in cases whereby no colonies were detected following antimicrobial treatment, the value of 0.5 colony-forming units was used to calculate LR values. Therefore LR values of 5.07 (highlighted in bold) indicate that no colonies of bacteria were detected following antimicrobial treatment and sampling. The Mean Log₁₀ density of untreated controls was calculated (± standard deviation).

	<i>P. aeruginosa</i>											
	24-Hour Biofilms						72-Hour Biofilms					
Treatment Time	1		5		15		1		5		15	
Antimicrobial	LR	Kill (%)	LR	Kill (%)	LR	Kill (%)	LR	Kill (%)	LR	Kill (%)	LR	Kill (%)
Cleancert	2.86 ± 0.92	99.86	2.44 ± 2.32	99.63	5.07	99.99	0.84 ± 0.98	85.61	3.92 ± 1.21	99.99	5.07	99.99
Orotol MD555	5.07	99.99	5.07	99.99	5.07	99.99	5.07	99.99	5.07	99.99	5.07	99.99
Orotol Plus	5.07	99.99	5.07	99.99	5.07	99.99	5.07	99.99	5.07	99.99	5.07	99.99
Asepticcate (4%)	0.77 ± 0.08	83.018	1.68 ± 0.05	97.92	2.31 ± 0.10	99.51	2.10 ± 0.05	99.22	2.35 ± 0.02	99.56	1.31 ± 0.12	95.07
Untreated control	Mean Log ₁₀ density of 7.62 ± 0.03 LR - 0 Kill (%) - 0						Mean Log ₁₀ density of 7.83 ± 0.15 LR - 0 Kill (%) - 0					

Table 6B. Log reduction (LR) and percentage kill (%) values of *P. aeruginosa* biofilms.

Biofilms were cultured for 24 or 72 hours in a CDC biofilm reactor before treatment with Cleancert (neat), Orotol MD555 (neat), Orotol Plus (neat) or Asepticcate (Neat 4%) for 1, 5 or 15 minutes. Samples were performed in duplicate. Mean Log values were calculated and the standard error (SE) of the mean LR was determined (LR ± SE). Given that the log of zero is mathematically undefined, in cases whereby no colonies were detected following antimicrobial treatment, the value of 0.5 colony-forming units was used to calculate LR values. Therefore LR values of 5.07 (highlighted in bold) indicate that no colonies of bacteria were detected following antimicrobial treatment and sampling. The Mean Log₁₀ density of untreated controls was calculated (± standard deviation).

Summary of Results 2

- Orotol Plus and Orotol MD555 were effective on all 24-hour and 72-hour biofilms after 1 minute of contact time, with no bacterial colonies detected following treatment.
- Cleancert was effective on all biofilms after 15 minutes of contact time, with no bacterial colonies detected after treatment.
- Asepticate (4%) did not completely kill *S. aureus* and *P. aeruginosa* biofilms after 15 minutes of treatment.

Appendix 1

Neutralisers

Antimicrobial	Neutraliser Recipe
Cleancert	20g Nutrient Broth, 1.5g Lecithin, 15g Tween-80, 5g Sodium thiosulphate, 0.5g L-Histidine, 485ml Distilled water, 3ml Phosphate buffer
Orotol Plus	20g Nutrient Broth, 1.5g Lecithin, 15g Tween-80, 5g Sodium thiosulphate, 0.5g L-Histidine, 485ml Distilled water, 3ml Phosphate buffer
Orotol MD555	20g Nutrient Broth, 1.5g Lecithin, 15g Tween-80, 5g Sodium thiosulphate, 0.5g L-Histidine, 485ml Distilled water, 3ml Phosphate buffer
Tetrasodium EDTA	0.5M Calcium Chloride in distilled water