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ORIGINAL ARTICLE

Rapid and quantitative automated measurement of bacteriophage activity against cystic fibrosis isolates of *Pseudomonas aeruginosa*

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bacteriophage, lytic activity, *Pseudomonas aeruginosa*.

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Abstract

Introduction: *Pseudomonas aeruginosa* is an opportunistic pathogen and is the main cause of respiratory infection in cystic fibrosis patients. Most strains prevalent within the UK are resistant to two or more antibiotics leading to the search for new therapeutic strategies including the use of bacteriophages.
Methods and Results: The infectivity of four bacteriophages was increased using an enhancement protocol based on the use of pomegranate rind extract. Their efficacy against 14 *Ps. aeruginosa* strains was measured using a qualitative streak test and a novel quantitative assay based on the Bioscreen C microbial growth analyzer. Streak test analysis illustrated an increase in the lytic activity of enhanced bacteriophages, whereas Bioscreen analysis showed that both enhanced and unenhanced bacteriophages failed to meet acceptable levels of activity in *c.* 50% of strains tested.
Conclusions: The quantitative Bioscreen C analyzer showed comparable but not identical results in phage activity and identified significant bacterial regrowth by 20 h postinfection.
Significance and Impact of the Study: With the resurgence of interest in bacteriophage therapy against infectious bacterial diseases, a rapid high throughput quantitative method for screening phage activity and bacterial resistance is required. The use of the Bioscreen C analyzer meets these criteria and was shown to be more stringent than the traditional streak test.

Introduction

Pseudomonas aeruginosa, an environmentally ubiquitous organism, is an opportunistic human pathogen that plays a key role in Cystic Fibrosis (CF) patients with around 80% of patients colonized by the age of 20 years (Saiman and Siegel 2004). With an increase in the level of antibiotic resistance over the past decade, there has been a renewed interest in alternatives to antibiotic therapy. Although in clinical use in Eastern Europe, bacteriophage therapy has only recently begun to attract attention in the West with a number of new products being licenced or currently in clinical trials (Merabishvili *et al.* 2009).

To successfully produce whole phage based therapeutics, a number of criteria must be met. These include the ability to infect a range of bacterial strains as well as the ability to reduce the overall level of infection (Hanlon 2007). One of two distinct approaches may be undertaken to create such a phage preparation: genetic modification (GM) of the phage genome to broaden specificity and to increase activity, or the selective breeding of phage, isolating the most infectious. The former of these two approaches is undoubtedly attractive allowing the ability to not only increase the rate and overall level of bacterial kill but also to provide genes that when expressed would enhance phage virulence, e.g., biofilm penetration through the production of an alginate lyase (Hanlon *et al.* 2001). However, products based on genetically modified (GM) phages might be more difficult to bring to market due to the level of regulation surrounding GM organisms. The latter approach on the other hand allows for the selection of more virulent phages on the basis of infectivity (Jassim *et al.* 1995).

The activity of phages is generally measured using traditional plating on agar of target bacteria where infection

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will be identified by plaque production (Adams 1959). Plaque morphologies and counts, however, vary yielding a subjective end point. The traditional streak assay, which relies on such a protocol is limited in its capacity and does not provide evidence for the emergence of bacterial resistance and re-growth (Merabishvili *et al.* 2009). Both phage induced lysis and bacterial re-growth can be followed by turbidometric measurements, offering an alternative and potentially high throughput method (Maillard *et al.* 1996).

In the present study, the efficacy of a novel high throughput method for measuring phage activity was tested against four *Ps. aeruginosa* phages and 14 strains of *Ps. aeruginosa* including isolates from CF patients. Phage activity was concurrently tested with a traditional streak test.

Materials and Methods

Host cells and bacteriophage cultures

Bacterial strains were kindly provided by Dr E Mahenthiralingam (School of Biosciences, Cardiff University, Cardiff, UK) (Table 1). *Ps. aeruginosa* PAO1 was obtained from the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, UK). *Ps. aeruginosa* NCTC 10332 (reference strain) was obtained from the Health Protection Agency (London, UK). Four bacteriophages, C10176-Large (C10176-L), C10176-Small (C10176-S), GL-1 and L-Phage-Medium (LP-M), were kindly provided by Prof. GW Hanlon (School of Pharmacy, University of Brighton, Brighton, UK). Bacterial strains and bacteriophages were stored at −80°C in 10% glycerol. Bacterial cultures were routinely prepared in tryptone soy broth (TSB; Oxoid Ltd, Cambridge, UK) and incubated at 37°C for 24 h. Cultures were centrifuged at 2600 ***g***, resuspended in tryptone sodium chloride (TSC; 1 g l^{-1} tryptone, 9·25 g l^{-1} NaCl) to a standardized optical density at 600 nm (OD_{600}) corresponding to 1–3 × 10^8 CFU ml^{-1}. Bacteriophage suspensions were routinely prepared using the soft overlay agar method (Adams 1959) with *Ps. aeruginosa* PAO1 used as the host. Five ml of phosphate buffered saline (PBS; Sigma, Dorset, UK) was used to remove the sloppy agar layer containing the phage. The phage suspension was then centrifuged at 11 000 ***g*** for 15 min at 4°C and passed through 0·45 and 0·2 μm membrane filters (Millipore, Cork, Ireland).

Preparation of pomegranate rind extract

Pomegranate rind extract (PRE) was kindly provided by David Houston (Welsh School of Pharmacy, Cardiff University, Cardiff, UK) and prepared as described in Stewart *et al.* (1998). Briefly, PRE was diluted by adding 1·3 ml of PRE in 8·7 ml of λ buffer immediately prior to use and 8·3 ml added to 16·7 ml of a freshly prepared ferrous sulfate solution (4·8 mmol l^{-1} $FeSO_4.7H_2O$). The solution was protected from light.

Enhancement of *Ps. aeruginosa* bacteriophage activity

The phage enhancement principle used in this study is based on the selection of the more virulent phage on the basis of invasion time. The enhancement protocol was adapted from Jassim *et al.* (1995) and is shown in Fig. 1.

Table 1 *Pseudomonas aeruginosa* strains used in this investigation

Strain name	Source	Comment
NCTC 10332	NCTC	Also known as ATCC 10145. Quality control strain for API products. Not producing alginate (Edwards and Saunders 2001)
PAO1	Clinical, nonCF	*Ps. aeruginosa* genome sequencing strain (Stover *et al.* 2000)
C3652*	CF	Epidemic Manchester CF strain type (Jones *et al.* 2001)
C3719*	CF	Epidemic Manchester CF strain type (Jones *et al.* 2001)
C3786*	CF	Melbourne *Ps. aeruginosa* CF strain – unique (Armstrong *et al.* 2003)
LES 400*	CF	Liverpool epidemic strain- Hypervirulent Strain predominant epidemic strain in the UK (Salunkhe *et al.* 2005; Fothergill *et al.* 2007)
Midlands-1-9245 (Mids-1)	CF	Midland 1 epidemic strain
P8959*	CF	Liverpool epidemic strain- predominant epidemic strain in the UK (Fothergill *et al.* 2007)
C1913*	CF	A55 unique- Unique genotype from Vancouver patients (Lewis *et al.* 2005)
C2238♦	CF	A61 unique- Unique genotype from Vancouver patients (Lewis *et al.* 2005)
C2846♦	CF	A55 unique-Unique genotype from Vancouver patients (Lewis *et al.* 2005)
C3597♦	CF	Nonepidemic Manchester CF strain type (Jones *et al.* 2001)
C4503♦	CF	A55 unique- Unique genotype from Vancouver patients (Lewis *et al.* 2005)
PAK-SR	Research Strain	Streptomycin^R parent of Fla-/Pil- mutants of PAK. Sm50

Strains indicated by * are epidemic CF strains. ♦ nonepidemic CF strains.

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The initial exposure time (referred to as enhancement time) was 15 min and subsequently shortened to 10, 5 and 2·5 min.

Streak test assay

The following methodology was adapted from (Merabishvili *et al.* 2009). Ten microlitres of bacterial suspension (*c.* 1×10^8 CFU ml^{-1}) was streaked over the surface of a 100 × 100 mm square petri dish (Sterilin Ltd, Caerphilly, UK) that was previously divided into a 5 × 5 grid and air dried at room temperature for 15 min. Ten microlitres of bacteriophage suspension (*c.* 1×10^7 PFU ml^{-1}) was then spotted onto each intersection and air dried at room temperature for 1 h. PBS was used as a negative control. Plates were incubated at 37°C for 3 h and a primary assessment for activity was made on the basis of the extent of phage lysis. A secondary reading was performed after a further incubation for 18 h at room temperature and a score of '0' (no plaque) to '+5' (confluent lysis) was attributed (Merabishvili *et al.* 2009) (Fig. 2). Ten separate experiments were conducted and a final score out of a maximum value of 50 was calculated.

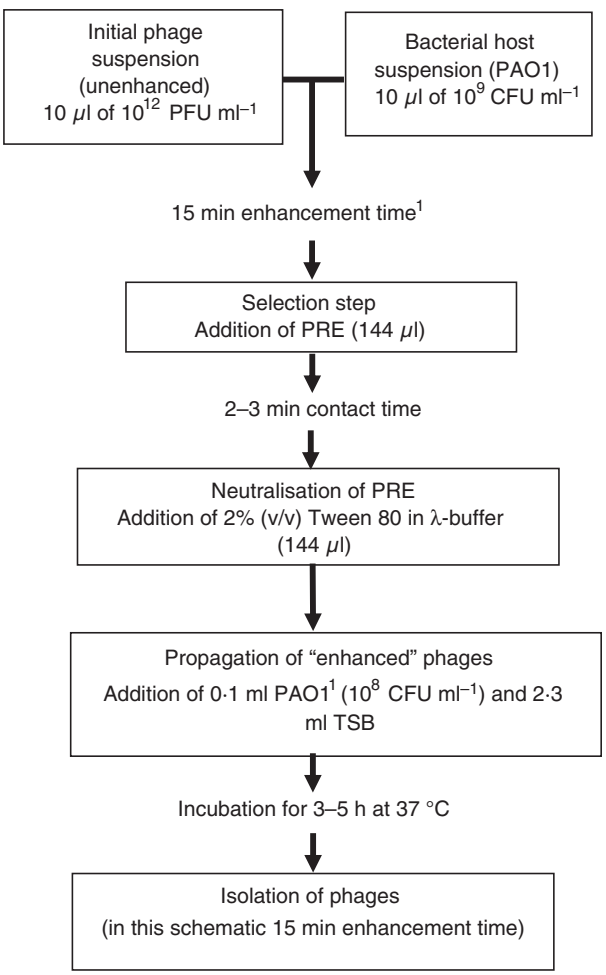

Figure 1 Schematic representation of bacteriophage enhancement using pomegranate rind extract. [1]:Contact time between phages and bacterial suspension varied (i.e. enhancement time of 2·5, 5, 10 and 15 min were used; see text).

Bioscreen assay

Three hundred and fifty microlitres of a bacterial suspension in TSB (*c.* 1×10^8 CFU ml^{-1}) was added to each well of a 100 well honeycomb plate (Oy Growth Curves AB Ltd, Helsinki, Finland) with TSB used as a negative control. To each well, 50 µl of standardized bacteriophage suspension (10^{11} PFU ml^{-1}) was added. This phage/bacterial cell ratio was determined in preliminary experiments to give the most appropriate phage activity, without directly lysing the bacterial cell on initial contact, thus allowing phage replication inside the host. To increase the reproducibility of the reading, a total of 10 wells for each phage were inoculated for each honeycomb plate. The plates were then incubated at 37°C for 20 h and turbidity recorded using a wideband filter (420–580 nm) with readings taken every 15 min in a Bioscreen C analyzer (Oy Growth Curves AB Ltd, Helsinki, Finland). Each reading was preceded by a 10 s shaking cycle. A total of three independent experiments were performed using fresh bacterial cultures and phage cultures

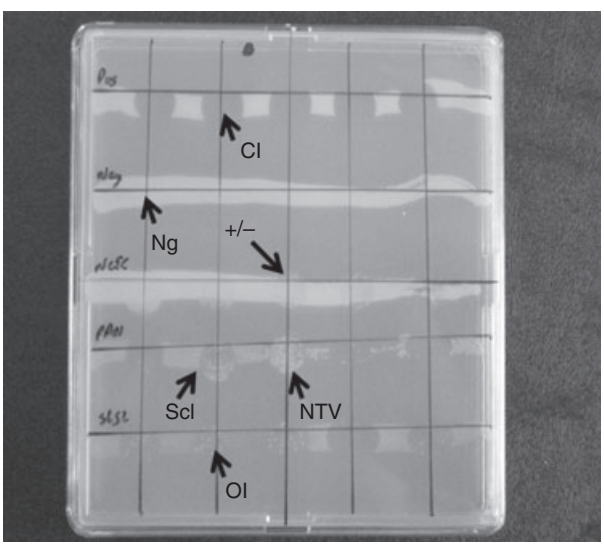

Figure 2 Example of a phage streak test plate at secondary reading stage. Cl – confluent lysis (+5), Ol – overgrowth, bacterial line completely broken, presence of singular bacterial colonies on spot (+4), Scl – Semi-confluent lysis- bacterial line not completely broken (+3), NTV – Multiple small phage plaques (+2), +/– Bacterial line just affected i.e. little observable disruption to bacterial line (+1) and Ng-negative (0).

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for each repeat. Once completed, the mean optical density (out of 10 wells) for each sample at each sampling time was calculated. Bacterial number at 8 and 20 h incubation time was calculated using a standard graph plotting optical density reading *vs* CFU ml^{-1}. Reduction in bacterial number (expressed as Log_{10} reduction) following phage exposure was calculated by comparing bacterial number recovered after 8 and 20 h incubation from wells exposed to phage and those not exposed to phages (control) (Fig. 3).

For this study, phage activity with the Bioscreen C analyser was measured against set 'acceptance' values attributed to two main parameters: (i) a ≥ 2 log_{10} decrease in bacterial number after 8 and 20 h (i.e. measurement of bactericidal efficacy) and (ii) a time of ≥480 min to reach a $OD_{420–580}$ of 0·1 above the original $OD_{420–580}$ (measurement of bacterial re-growth postphage infection) (Fig. 3 and Table 2). Other parameters were also calculated but did not contribute to the assessment of phage efficacy. These included the measurement of phage initial lytic activity indicated by a decrease in $OD_{420–580}$ within 30 min of phage addition (Fig. 3) and the calculation of the lytic slope, which indicates the rate of bacterial lysis. The attribution of set 'acceptance' values (Table 2) was based on a review of the literature for phage activity (Hanlon *et al.* 2001; Atterbury *et al.* 2007; Fu *et al.* 2010) and antibiotic dosing times (Van Zanten *et al.* 2007; McCoy *et al.* 2008).

Statistical analysis

Data were analysed for significant differences using a one-way ANOVA in Minitab 15 (Minitab Ltd, Coventry, UK).

Results

Assessment of phage activity using the streak test method

Phage activity was enhanced against *Ps. aeruginosa* PAO1. Phages showing an increase in activity were then tested against the different clinical isolates.

The streak test showed an increase in the level of lytic activity of the four phages against *Ps. aeruginosa* PAO1 (Table 3). The largest level of increased lytic activity against PAO1 was after the first enhancement step of 15 min (data not shown). The further enhancement steps following 10, 5 and 2·5 min contact time lead to smaller increases in activity (data not shown). Ultimately, the maximum level of lytic activity against PAO1 was reached after the final enhancement time of 2·5 min for all phages, except for GL-1, for which a maximum activity was reached after the enhancement time of 5 min (Table 3). When used against all bacterial isolates, the highest increase in activity was observed for C10176-L and LP-M and the smallest for GL-1. For some bacterial strains, notably for the CF

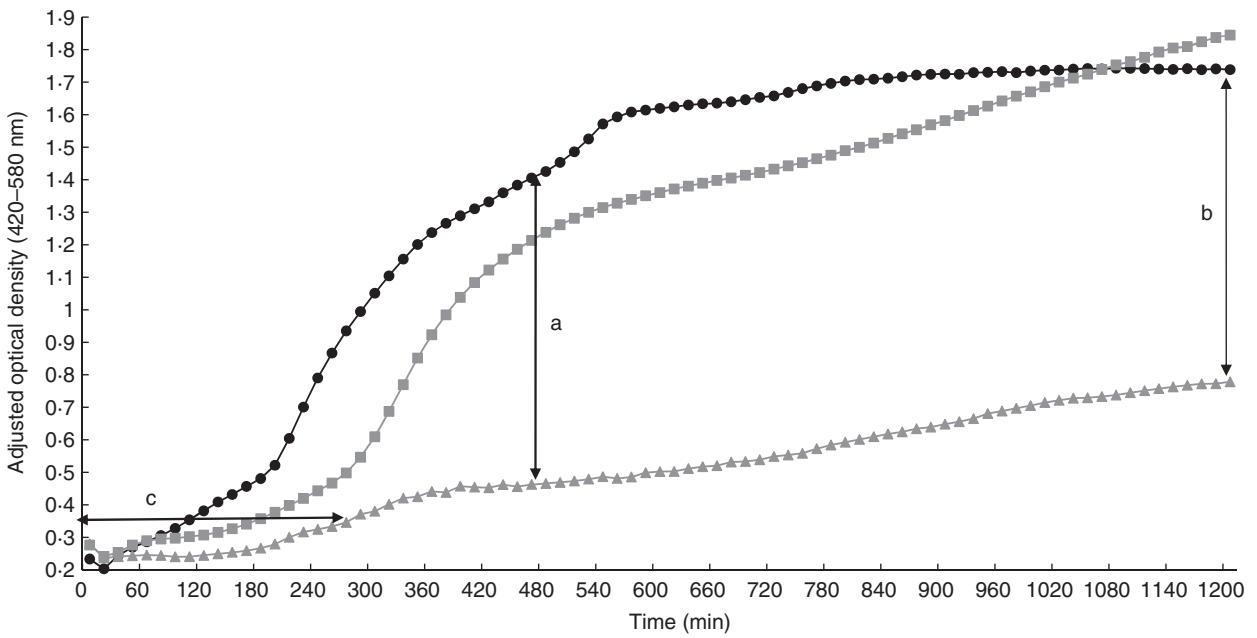

Figure 3 Representative change in bacterial growth patterns of *Pseudomonas aeruginosa* strain C1913 following treatment with original and enhanced C10176-S bacteriophage. (a and b) bacterial reduction at 8 and 20 h respectively. (c) time taken for the $OD_{420–580}$ to increase by 0·1 above the initial $OD_{420-580}$. Phage treated samples are the mean of three replicates; untreated samples are the mean of six replicates. (●) Untreated Cultures; (■) nonPRE treated phage and (▲) PRE treated phage.

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Table 2 Summary of the lytic assessment criteria of *Pseudomonas aeruginosa* bacteriophages from Bioscreen C assessment

		Evaluation criteria			Additional criterion
		Log_{10} reduction		Time* (min)	
		8 hours	20 hours		Lysis† (min)
'Acceptance' value‡		≥2	≥2	≥480	≤30
Phage	Treatment	No. of bacterial strains meeting the acceptance value out of 14			
GL-1	Non-PRE	3	3	8	7
C10176-S	treated	3	4	8	7
C10176-L		6	6	8	7
LP-M		7	10	8	7
GL-1	PRE	7	7	7	4
C10176-S	treated	6	6	8	4
C10176-L		6	7	6	4
LP-M		7	6	6	4

*Time taken to increase $OD_{420–580}$ by 0·1 above the original $OD_{420–580}$.
†Time taken to induce a lytic slope.
‡Set 'acceptance' value (see text).

Table 3 Comparison of lytic activity of phages against *Pseudomonas aeruginosa* PAO1 between the streak test method and the Bioscreen C analyzer

		Bioscreen C analyser			
		Log_{10} reduction (CFU ml^{-1})			
Phage treatment	Streak test (score out of 50)	8 hours	20 hours	*P*-values*	Time† (minutes)
C10176-L					
None‡	20	2·06 ± 0·50	2·28 ± 0·89	0·724	>1200
PRE§ (2·5)¶	48	3·14 ± 0·10	3·20 ± 0·46	0·834	>1200
C10176-S					
None	29	2·04 ± 0·52	2·31 ± 0·83	0·662	>1200
PRE (2·5)	47	3·14 ± 0·10	3·23 ± 0·52	0·668	>1200
GL-1					
None	20	1·95 ± 0·51	1·24 ± 1·10	0·367	815 ± 670
PRE (5)	46	2·66 ± 0·25	2·72 ± 0·13	0·712	>1200
PRE (2·5)	43	3·15 ± 0·11	3·18 ± 0·40	0·905	>1200
LP-M					
None	30	2·01 ± 0·45	2·28 ± 0·89	0·667	>1200
PRE (2·5)	45	3·01 ± 0·30	2·97 ± 0·55	0·917	>1200

Data are based upon the means of three replicates.
**P*-values showing differences in log_{10} reduction at 8 and 20 h.
†Time taken to increase $OD_{420–580}$ by 0·1 above the original $OD_{420–580}$.
‡None: phage not treated with PRE (i.e. original phage).
§PRE: phage exposed to PRE.
¶(time): phage-bacteria contact time (min) before exposure to PRE (see text).

epidemic strain Midlands-1, no increase in phage activity was observed, whereas only LP-M phage showed some increase in activity against CF nonepidemic strain C4503 (Tables 4–7). Overall, C10176-L and LP-M showed increased levels of activity against 11 strains, C10176-S against 10 strains and GL-1 against six strains (Tables 4–7). Surprisingly, all enhanced phages showed decreased levels of activity against Midlands-1 (data not shown). Finally, the nonepidemic CF bacterial strains were overall more susceptible to the enhanced phages when compared to epidemic strains.

Assessment of phage activity using the Bioscreen C method

When PAO1 was challenged with phages, a lytic slope was observed within 30 min of the initial inoculation for original phages and immediately upon exposure for

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Table 4 Summary table of Bioscreen C Data for *Pseudomonas aeruginosa* strains exposed to C10176-L phage

	Original phage			Enhanced phage		
		Bioscreen C analyser			Bioscreen C analyser	
Bacterial strain	Streak test (score out of 50)	Log_{10} reduction*	Time† (minutes)	Streak test (score/50)	Log_{10} reduction*	Time† (minutes)
10332	10	0·90 ± 0·78	205 ± 10	21 (10)‡	1·90 ± 0·65	180 ± 40
C3652	10	0·94 ± 0·14	590 ± 85	36 (10)	1·06 ± 0·15	1005 ± 340
C3719	0	0·85 ± 0·05	565 ± 10	25 (10)	1·05 ± 0·15	>1200
C3786	44	2·58 ± 1·05	130 ± 10	–§	2·74 ± 0·60	140 ± 10
LES-400	10	1·09 ± 0·18	1155 ± 80	29 (2·5)	0·88 ± 0·27	860 ± 280
Mids-1	10	ND¶	105 ± 45	–	ND	140 ± 25
P8959	10	1·99 ± 0·20	1180 ± 35	27 (10)	1·72 ± 0·46	770 ± 285
C1913	0	4·36 ± 1·79	345 ± 55	22 (2·5)	3·57 ± 2·24	240 ± 50
C2238	0	0·59 ± 0·07	865 ± 135	49 (5)	0·59 ± 0·14	790 ± 325
C2846	20	2·39 ± 0·95	**305 ± 30**	24 (10)	3·04 ± 0·83	**205 ± 40**
C3597	47	3·78 ± 0·82	**570 ± 210**	50 (10)	2·47 ± 0·09	**185 ± 40**
C4503	20	0·07 ± 0·04	130 ± 10	–	0·95 ± 1·70	380 ± 440
PAK-SR	16	2·90 ± 0·22	1085 ± 150	40 (15)	3·05 ± 0·26	1000 ± 165

Data shown are the mean of three replicates. Bold typeface indicates significant differences.
*Log_{10} reduction in bacterial number at 8 h.
†Time taken to increase $OD_{420–580}$ by 0·1 above the original $OD_{420–580}$.
‡(time): phage-bacteria contact time (min) before exposure to PRE (see text).
§Dashes indicate no increase or decrease in lytic activity.
¶ND: no reduction in bacterial content determined.

Table 5 Summary table of Bioscreen C Data for *Pseudomonas aeruginosa* strains exposed to C10176-S phage

	Original phage			Enhanced phage		
		Bioscreen C analyser			Bioscreen C analyser	
Bacterial strain	Streak test (score out of 50)	Log_{10} reduction*	Time† (minutes)	Streak test (max. 50)	Log_{10} reduction*	Time† (minutes)
10332	14	0·47 ± 0·78	220 ± 30	27 (15)‡	1·64 ± 1·20	180 ± 25
C3652	30	0·97 ± 0·24	900 ± 520	40 (10)	0·95 ± 0·37	558 ± 200
C3719	30	0·89 ± 0·24	890 ± 540	31 (15)	0·77 ± 0·31	485 ± 170
C3786	9	**0·87 ± 1·31**	**100 ± 25**	45 (2·5)	**3·32 ± 0·80**	**145 ± 10**
LES-400	14	0·91 ± 0·24	805 ± 280	30 (2·5)	1·50 ± .121	980 ± 330
Mids-1	18	ND§	95 ± 30	–¶	ND	140 ± 25
P8959	22	1·96 ± 0·2	**1100 ± 85**	–	1·81 ± 0·08	**825 ± 145**
C1913	30	**1·50 ± 0·33**	200 ± 30	–	**4·49 ± 1·05**	425 ± 285
C2238	8	0·56 ± 0·11	805 ± 50	40 (10)	0·62 ± 0·13	865 ± 340
C2846	25	0·76 ± 0·67	215 ± 25	30 (10)	2·73 ± 1·52	215 ± 50
C3597	2	3·73 ± 0·78	500 ± 280	46 (5)	3·39 ± 0·59	895 ± 295
C4503	30	ND	140 ± 10	–	0·81 ± 1·63	370 ± 435
PAK-SR	24	2·84 ± 0·26	1095 ± 160	46 (5)	3·04 ± 0·35	1025 ± 100

Data shown are the mean of three replicates. Bold typeface indicates significant differences.
*Log_{10} reduction in bacterial number at 8 h.
†Time taken to increase $OD_{420–580}$ by 0·1 above the original $OD_{420–580}$.
‡(time): phage-bacteria contact time (min) before exposure to PRE (see text).
§ND: no reduction in bacterial content determined.
¶Dashes indicate no increase or decrease in lytic activity.

enhanced phages. However, there was no significant ($P \geq 0·05$) change in the rate of $OD_{420–580}$ decrease per minute between original and enhanced phages (data not shown). A lytic slope was not necessarily observed with all CF isolates exposed to the parent or enhanced phages (data not shown). Where a decrease $OD_{420–580}$ was noted (against *Ps. aeruginosa* LES400, P8959 and PAK-SR), there was no statistically significantly

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Table 6 Summary table of Bioscreen C Data for *Pseudomonas aeruginosa* strains exposed to GL-1 phage

	Original phage			Enhanced phage		
		Bioscreen C analyser			Bioscreen C analyser	
Bacterial strain	Streak test (score out of 50)	Log_{10} reduction*	Time† (minutes)	Streak test (max. 50)	Log_{10} reduction*	Time† (minutes)
10332	35	0·99 ± 0·74	230 ± 35	–‡	2·09 ± 0·98	180 ± 25
C3652	34	1·23 ± 0·17	885 ± 295	38 (10)§	0·90 ± 0·20	505 ± 140
C3719	46	0·93 ± 0·17	585 ± 130	–	0·79 ± 0·20	455 ± 160
C3786	44	0·77 ± 0·67	110 ± 30	–	2·72 ± 1·43	145 ± 10
LES-400	30	1·09 ± 0·13	**1185 ± 25**	34 (2·5)	0·97 ± 0·21	**940 ± 80**
Mids-1	30	ND¶	**85 ± 20**	–	ND	**140 ± 25**
P8959	35	1·79 ± 0·34	905 ± 175	–	1·95 ± 0·07	915 ± 25
C1913	6	**3·30 ± 0·85**	310 ± 20	22 (2·5)	**5·06 ± 0·33**	345 ± 90
C2238	23	0·51 ± 0·08	765 ± 70	40 (10)	0·57 ± 0·16	840 ± 240
C2846	35	**0·97 ± 0·50**	240 ± 15	–	**2·90 ± 0·08**	345 ± 275
C3597	50	3·80 ± 0·78	560 ± 185	–	2·98 ± 0·19	495 ± 300
C4503	34	ND	140 ± 20	–	0·90 ± 1·52	350 ± 400
PAK-SR	30	2·78 ± 0·23	**>1200**	46 (2·5)	2·98 ± 0·31	**1020 ± 40**

Data shown are the mean of three replicates. Bold typeface indicates significant differences.

*Log_{10} reduction in bacterial number at 8 h.

†Time taken to increase $OD_{420–580}$ by 0·1 above the original $OD_{420–580}$.

‡Dashes indicate no increase or decrease in lytic activity.

§ (time): phage-bacteria contact time (min) before exposure to PRE (see text).

¶ND: no reduction in bacterial content determined.

difference ($P \geq 0·05$) between original and enhanced phages (data not shown). In the strains where no lytic slope was observed (e.g. C1913), the growth pattern of the bacterial isolates was dramatically altered by the phage (example in Fig. 3) and the final $OD_{420–580}$ at 20 h was lower following exposure to enhanced phages. It has to be noted that as our protocol is based on OD reading, bacterial cell debris following lysis might have contributed somewhat to the OD values recorded and as such might have masked initial cell lysis following initial phage interaction/replication in their host. However, the presence of cell debris cannot explain solely the dramatic change in bacterial growth pattern recorded following phage treatment.

When the phage bactericidal activity to PAO1 was evaluated, enhanced phages showed a significantly higher ($P \leq 0·05$) level of bacterial reduction compared to their original counterparts at both 8 and 20 h (Table 3). There was no significant difference ($P \geq 0·05$) in the reduction of bacterial number after 8 and 20 h incubation (Table 3). There was, however, a significant difference ($P = 0·034$) between the efficacy of the original and enhanced phages at 8 h and these results were in accordance to those obtained from the streak test (Table 3). In the case of GL-1 where a 5 min PRE treatment yielded the highest score by streak test, there was no significant difference ($P \geq 0·05$) in phage activity between the 5 and 2·5 min enhancement time (Table 3) when measured with the Bioscreen. Furthermore, in PAO1, there was no significant difference in bacterial re-growth following original and enhanced phage exposure (Table 3).

There was generally no significant difference ($P \geq 0·05$) in activity between original and enhanced phages at 8 h exposure against CF isolates (Tables 4–7), with the exception of *Ps. aeruginosa* C3786 and C1913 exposed to C10176-S (Table 4) and C1913 and C2846 challenged with GL-1 (Table 6). After 8 h incubation at 37°C, all bacterial strains showed a reduction in bacterial number when exposed to original or enhanced phages with the exception of Midlands-1.

Following exposure to phages, all bacterial strains showed an eventual increase in $OD_{420–580}$ (Tables 4–7). This $OD_{420–580}$ increase is indicative of bacterial re-growth and potentially indicates the presence of resistant bacteria to the phage. However, the extent of $OD_{420–580}$ increase varied between bacteria/phage combinations. In the majority of cases, the time taken to increase the $OD_{420–580}$ by 0·1 above the initial $OD_{420–580}$ was reduced when bacteria were exposed to enhanced phage (Tables 4–7).

Comparison of activity between the streak test and the Bioscreen C

Overall, there was some correlation between the streak test assay and the Bioscreen C assay against PAO1, where

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Table 7 Summary table of Bioscreen C Data for *Pseudomonas aeruginosa* strains exposed to LP-M phage

	Original phage			Enhanced phage		
		Bioscreen C analyser			Bioscreen C analyser	
Bacterial strain	Streak test (score out of 50)	Log_{10} reduction*	Time† (minutes)	Streak test (max. 50)	Log_{10} reduction*	Time† (minutes)
10332	32	0·87 ± 0·56	200 ± 20	–‡	2·01 ± 0·80	170 ± 30
C3652	22	1·08 ± 0·02	880 ± 280	35 (10)§	0·92 ± 0·32	505 ± 275
C3719	20	0·95 ± 0·24	**>1200**	31 (10)	0·76 ± 0·29	**450 ± 250**
C3786	40	3·03 ± 1·02	130 ± 20	42 (2·5)	2·70 ± 0·99	140 ± 10
LES-400	30	1·08 ± 0·19	1155 ± 80	–	0·91 ± 0·23	940 ± 320
Mids-1	10	ND¶	115 ± 40	–	ND	135 ± 15
P8959	20	2·03 ± 0·17	>1200	26 (10)	1·76 ± 0·28	830 ± 285
C1913	0	5·57 ± 0·45	545 ± 115	22 (10)	4·84 ± 0·86	350 ± 205
C2238	20	0·77 ± 0·09	>1200	50 (5)	0·51 ± 0·17	705 ± 370
C2846	43	3·00 ± 0·36	**305 ± 10**	50 (10)	3·01 ± 0·31	**200 ± 40**
C3597	43	3·90 ± 0·82	**605 ± 85**	50 (10)	2·66 ± 0·26	**220 ± 60**
C4503	10	ND	130 ± 10	21 (10)	0·83 ± 1·54	335 ± 360
PAK-SR	4	2·95 ± 0·26	995 ± 75	42 (2·5)	2·85 ± 0·33	950 ± 55

Data shown are the mean of three replicates. Bold typeface indicates significant differences.

*Log_{10} reduction in bacterial number at 8 h.

†Time taken to increase $OD_{420–580}$ by 0·1 above the original $OD_{420–580}$.

‡Dashes indicate no increase or decrease in lytic activity.

§ (time): phage-bacteria contact time (min) before exposure to PRE (see text).

¶ND: no reduction in bacterial content determined.

all enhanced phages with the exception of GL-1 showed the highest reduction of bacterial number with the Bioscreen C (Table 3). This fact corresponded to the highest scores achieved with the streak test (Table 3). However, with enhanced GL-1, the largest reduction in bacterial number was obtained after an enhancement time of 2·5 min, compared to the highest score on the streak test that was obtained after a 5 min enhancement (Table 3). When comparing the time taken for PAO1 to re-grow to an $OD_{420–580}$ value of 0·1 higher than the initial $OD_{420–580}$, it was found that for all phages, except GL-1, bacteria treated with both original and enhanced phages took >1200 min to reach this value (Table 3).

When the activity of phages was assessed against CF isolates, the level of correlation between the streak test and the reduction of bacterial number from the Bioscreen C method depended upon phage/bacterial strain. A good correlation was observed with LP-M and two strains, C10176-L and five strains, GL-1 and six strains and C10176-S and seven strains (Tables 4–7). For all the other phage/bacteria combinations, however, the level of reduction in bacterial number at 8 h was not significantly different ($P \geq 0·05$) when exposed to the original or the enhanced phages.

When phage performance was compared to the set 'acceptance' values, it was observed that an 'acceptable' status was achieved against 50% or less strains (Table 2). However, enhanced phages appeared to have a better activity than the original phage although bacterial re-growth was faster in many bacteria.

Discussion

With the renewed interest in bacteriophage therapy for a variety of conditions ranging from *Ps. aeruginosa* infections in cystic fibrosis patients to the use of bacteriophages to reduce the level of *Salmonella* colonization of broiler chickens (Atterbury *et al.* 2007), there is a need for a high throughput method, which is both sensitive and quantitative.

Although a successful phage product does not hinge on the initial screening of activity, the selection of phages may be enhanced by efficient and quantitative screening methods to ensure maximum activity against the widest host range. Here, we have shown that both the streak plate method and the Bioscreen method produced similar results, at least with phages showing a large increase in activity. Although the streak test provides adequate qualitative data for initial screening in the form of a single number, it does not provide the quantitative data that would be needed to select phages for practical applications. Conversely, the data generated by the Bioscreen C analyser allows for the assessment of multiple criteria. These criteria, however, should not be taken individually, but should instead be combined, and phages selected on the basis of increased characteristics between all criteria combined. Our assay relied on the calculation of bacterial

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number from a pre-established OD/TVC graph. Such extrapolation of bacterial number has its limitation as the OD/TVC graph is not linear above an OD value of *c.* 0·8. At best, this limitation means that our bacterial number calculated from OD value >0·8 might have been underestimated. However, the conversion of OD value in bacterial number, and the subsequent calculation of Log_{10} reduction, enables the direct comparison of phage activity between strains that show different growth kinetic to be established. Our method aimed to provide criterion to compare phage activity against multiple bacterial strains that might show different growth characteristic. Although, the conversion of OD values to bacterial number has its drawbacks, our results proved to be reproducible and enabled phage activity to be effectively compared.

In this manuscript, two methods were compared and some similarities were observed. It has to be noted that the parameters used in the two methodologies were different, notably the different phage: bacteria ratio, the incubation temperature following exposure and the media. These parameters might have contributed to the differences in results observed. The parameters used with the Bioscreen were developed in preliminary experiments and chosen as they provided the best results. The parameters used in the traditional streak test are those described in the literature and used routinely. The main objective of this paper was to report an automated method to improve on the traditional streak test. The main practical difference between the two methods remains with the number of bacteria/phage combination that can be tested. With the streak test, a maximum of four phage combinations and one set of controls (five replicates per bacterial line) against a single bacterial species could be tested in any one experiment. However, with the Bioscreen assuming the same number of replicates, a maximum of 18 phage combinations could be investigated showing that the Bioscreen has a higher throughput. In addition, the Bioscreen method provided important quantitative information on bacterial re-growth following phage exposure over a 20 h period.

In this study, four *Ps. aeruginosa* bacteriophages were confirmed to possess activity against multiple strains of *Ps. aeruginosa* including some of the more prevalent strains within the UK (Salunkhe *et al.* 2005; Fothergill *et al.* 2007). Result variability is most probably caused by the inherent variability that is associated with both bacterial and bacteriophage growth despite initial standardization of bacterial content. The differences in susceptibility of the phages may be a result in part to the binding efficacy of the phage to the target cell (Hart *et al.* 1994) and also the growth rates of the strains tested. The nature of the PRE treatment also accounts for a lack of increase in both rate and level of kill as it was originally designed to select bacteriophages on the basis of invasion time rather than other factors (Jassim *et al.* 1995).

The use of the Bioscreen C analyzer produced a quick real-time assessment of bacteriophage activity. It allows the rapid visualization of bacterial lysis demonstrating phage activity although measurement of the rate of lysis was shown not to be a reliable criterion for the comparison of lytic activity. Measurement of OD in the Bioscreen assay is automated and does not rely on human qualitative assessment on plaque formation as in the streak assay (Merabishvili *et al.* 2009) and was shown to be more stringent compared to the streak test and provided quantitative results within 8 h. In comparison, the streak test method can take up to 24 h (Merabishvili *et al.* 2009). The Bioscreen method could be further refined with the use of metabolic assays to assess the level of bacterial viability (Kuda and Yano 2003; Cerca *et al.* 2005) or potentially bioluminescent mutants (Marques *et al.* 2005; Jassim and Griffiths 2007; Thorn *et al.* 2007), which provide direct measurements of bacterial viability, ultimately increasing the sensitivity of this OD-based quantitative assay.

The set 'acceptance' values used in this study aim to ensure that selected phages possess acceptable properties to be used for therapy. The phages used in this study meet these criteria in 50% or less of strains tested, implying that their activity is not yet sufficient to be applied to therapeutics. These 'acceptance' values were set after a review of the literature and taken into account antibiotic dosing to prevent bacterial re-growth. It is conceivable that our values are too stringent and might need to be revised. To this aim the testing of phages that are currently undergoing licencing or already licenced for human applications would be of benefit.

The Bioscreen method is an *in vitro* set up and phage behaviour *in vitro* might be different from their behaviour *in situ*. The chemical and physical parameters used in the Bioscreen set up (e.g. growth media, temperature; % CO_2) can be modified to represent better conditions found in practice.

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