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Comparing assays for Clinical Phage Microbiology in biofilm

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INTRODUCTION

Failure of bacterial infections for current Antimicrobial treatment is a major burden on global health, estimating that by 2050 the leading cause of death will be antibiotic resistant infections (1). These non resolving infections are commonly associated with bacterial biofilm formation (2).

One promising solution for this problem is phage therapy, however, a major challenge in the use of phages for therapy is the personal matching of the best phage for patients' bacteria, with the most effective antibiotic combination against the target bacteria. Different phage and antibiotic combination can be assessed in various methods on planktonic bacteria, most common using an optical density plate reader in a process termed clinical phage microbiology (3).

Currently there is no validated reliable method to determine the biofilm effect of different phages
Herein we assessed 7 different assays: metabolic, genomic, microbiologic and different fluorescent stains and CFU counting, in the model bacterium pseudomonas aeruginosa PA14.

We aimed to identify an assay that will be sensitive enough to differentiate between 5 different pseudomonas aeruginosa targeting phages, and between different concentrations of the same phage, with the hypothesis that a system that will be sensitive enough to observe the greater effect of higher phage concentration on pre-grown biofilms, will be able to correctly identify difference between different phages.

METHODS

Bacterial Strains and Culture Conditions. Pseudomonas aeruginosa *PA14* (4), was used as model microorganism. Bacteria was grown in Luria-Bertani (LB, Oxoid Basingstoke, United Kingdom) broth in liquid or solid form (1.5% agar) at 37°C with shaking. Low shear force biofilm was grown as previously described (5), overight frown *PA14* was put in a desired well plate and incubated for 24 hours. Phages used in this study were previously isolated as part of the Israeli phage bank (6). The phage concentration was determined according to the standard PFU method
Determination of bacterial concetntration Bacterial concentrations were evaluated according to standard CFU method. Biofilm was washed with saline, and mechanically removed using a sterile tip. The content of each well was moved into an 1.5 ml tube, followed by sonication for 5 minutes. CFU methods was done.

Symcel calScreener biofilm was grown in designated activated Symcelmanifactured plates,as described. 20ul phages were added, and plastic leads were placed in the machines titanium wells , and sealed. Machine measurement started after a period of 1 hour due to the calScreener protocol. Data was analysed using the supplied software.
qPCR for eDNA Biofilm was grown as dsceibed above. 20 µl Phage or PBS for the time dseribed or overnight if not mentioned. The relative amounts of eDNA in the cell-free supernatants were determined by real-time PCR using specific primers for housekeeping genes rpoD and the ldhD genes. Real-time PCR was carried out using the iQ™ SYBR® Green Supermix, (Biorad, Hercules, California, United States), with a CFX96 Touch Real-Time PCR Detection System (Biorad, Hercules, California, United States), in a 25 µl final volume.
For determination of eDNA in changing CFU planktonic bacteria were grown iver night and treated with 6 dilutions of ceftazidime: 50, 25, 12.5, 6.25, 3.125, 1.5625 µg/ml, incubated overnight at 37°C with shaking. DNA was quantified using nanodrop (Thermo Fisher, Waltham, MA).
CV Biofilm containg 96 well plate was washed, then stained with 0.1% crystal violet solution (125 µl per well) and extracted with 30% acetic acid (100 µl per well), OD was determined in 550nm.
Fluorescent stainings Biofilm containing 96 well plate was washed and then stained with either Live/Dead cell viability kits, DAPI, Biologht 680 according to the manufacturer's instructions. The fluorescence emissions of the samples were detected by using confocal laser microscope (Carl Zeiss). DAPI, Biolight 680 were also assessed with a fluorescent plate reader every 20 minutes, at previously described configuration

RESULTS

Metabolic method : Symcels calScreener
The calScreener is an isothermal microcalorimetry instrument. It directly monitors the metabolic response in biological systems using bioactivity monitoring.

calScreener measures heat flow in each well in a 32 well plate, in a closed system the change in heat generation is contributed to metabolic change. When comparing 10^8 PFU applied phages on biofilm, reduction of 28.8% (PASA16) to 92% (PB2C) (*p-value* <0.01 Fig. 1). AUC of all 5 phages tested was higher when treated with lower phage PFU (*p-value*<0.01, Fig. 1). The released heat measured in uW from t=1h until t=2h was lower when PFU was higher in 4 out of 5 phages tested (Fig. 1B-E).

All wells tested have reached a decay due to anerobic conditions of growth in calScreener, and a limited amount of oxygen. In specific cases bacterial regrow and adaptation have been recognized (Fig. 1 A, B, D).
measurement starts after a period of 1 hour due to the calScreener protocol, demanding that post treatment, plate wells will be assembled into titanium wells, and their leads sealed using a screwdriver.

Genomic methods: external DNA (eDNA) quantitive PCR (qPCR) eDNA is relarsead from withing bacteria when they undergo lysis .It is isolated through 0.22 filtration of lysate.

qPCR of eDNA have shown a significant increase in eDNA presence for two pseudomonas housekeeping genes, *ldhD* and *rpoD*. for ldhD, 3 out of 5 phages tested have shown a significant reduction in cq when treated with higher phage concentration (*p-value*<0.01, Fig. 2A). *rpoD* control group has reached detection threshold in cq 18.25 cycle, while 10^8 PFU/ml phage group resulted from 15.69 cq (PB2C) to 17.54 cq (PB2F) (*p-value*<0.01, Fig. 2B). all 5 phages tested have shown a significant reduction in cq when treated with higher phage concentration (*p-value*<0.05, Fig. 2B).

We next analyzed time series experiment where replicates of the same biofilm treatment were filtered at different timepoints to assess for the kinetics of eDNA change over time.
We saw a significant reduction in cq between the phage treated biofilm to the control treated biofilm over time in both genes tested , from a about 1 hour (*p-value*<0.001, Fig. 2C-D).

We next correlated the lysis of different planktonic bacterial concentrations to the change in the amount of eDNA, a reduction of 1 log in CFU resulted in a cq of 18 cq, for *ldhD*, and 18.58 cq for *rpoD* (*p-value*<0.001, Fig. 2E).
We then found the minimal amount of DNA detected through this method, which was found to be 10^{-3}ng/mµ.
We found a linear correlation between DNA content and qPCR detection.

Sonication and CFU, Crystal Violet. Counting living cells, and biological mass

Both Colony Forming Unit (CFU) after sonication and Crystal Violet (CV) were not sensitive enough to differentiate between the activity of different concentration of phages (Fig. 3) both of them demand the termination of experiment and cannot be assessed real time, CFU demands the use of sonication to dissolve biofilm-cell living morphology, while CV assess biomass and not living cells

Fluorescent dyes: SYTO9 ("Live"), Propidium Iodide ("Dead"), Biolight 680, DAPI – Assessing living/lysed cells, extracellular matrix

Live dead stain did appear cytotoxic, but not phage toxic both Live staining (SYTO9) and Dead (propidium iodide) were able to different between phage concentrations tested of 4 out of 5 phages tested (different phages for each of the two dyes), at the endpoint, demanding the staining, confocal microscopy imaging and analysis (Fig. 3A-C).

DAPI, targeted staining DNA (example in Fig. 4), our hypothesis is that through bacterial lysis DNA is realesed and DAPI signal is increased. Our phage panel was assessed, stained with DAPI and read with 96 well plate reader. AUC significantly increases when increasing phage PFU in all 5 phages tested. Biologht 680 signal (example at Fig. 4) was reduced with phage application, reduced more with higher phage PFUs

Assay Type	Assay Name	Focused on bacterial cells/ extracellular matrix	Sensitivity[1]	Real time	Regrow[2]	Equipment and cost	Labor and experty	Test biofilm on target material?	Quantitative	Other
Metabolic	calScreener	Bacterial cells	High	Yes	Yes	Costly	1 hour, a short instruction is needed	Yes	Yes	Anerobic conditions
Genomic	Quantitative PCR (qPCR)	Bacterial cells	High	No[3]	No	Basic Lab Equipment	3 hours, Laborious	Yes	Yes	demands filtering of samples
Microbiological	Post Sonication Colony Forming Units (CFU)	Bacterial cells	Low	No[3]	No	Basic Lab Equipment	Minimal	Yes	Yes	
Microbiological	Crystal Violet staining (CV)	Extracellular matrix	Low	No[3]	No	Basic Lab Equipment	Minimal	Yes	Yes	
Fluorescent	Live Dead stain	Bacterial cells	Medium	No[3]	No	Confocal Microscopy	Laborious, 30 minutes per sample	Yes	Yes	
Fluorescent	DAPI stain	Bacterial cells	High	Yes	No	Fluorescent plate reader	Minimal	No	Yes	Could be combined
Fluorescent	Biolight 680	Extracellular matrix	High	Yes	No	Fluorescent plate reader	Minimal	No	Yes	

[1]The ability to differentiate between the same phage at different PFUs, and the ability to differentiate between different phages.
[2]The ability to observe bacterial grow after initial reduction.
[3]Real time is only possible if conducting a tme series experiment, as described in Fig. 2 C-D.

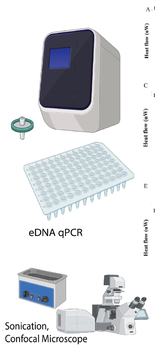

Figure 1. heat flow was measured using calScreener over 21 hours, comparing 5 different phages at a PFU of 10^8 and 10^6 on Pseudomonas aeruginosa biofilm. PASA16 phage (A), PB2C phage (B), D9 phage (C), PB2F phage (D), KLEIN 3 phage (E). measurement starts after a period of 1 hour due to the calScreener protocol, The results are the average of triplicates, presented as mean ± SD. AUC of the different phages at different concentrations (F). This shows that calScreener is sensitive shows lower AUC with higher phage PFU.

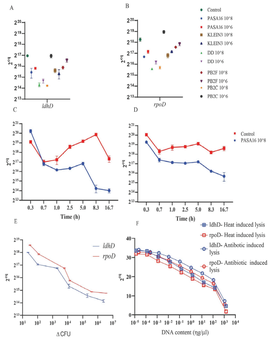

Figure 2. Comparison, using rtPCR, of 5 different phages at a PFU of 10^8 and 10^6 treated for 24 hours, and then their supernatant was filtered. Primers for housekeeping genes, *ldhD* (A), *rpoD* (B). The results are the average of triplicates, presented as mean ± SD. Time Series experiment with one representitive phage PB2C at a PFU of 10^8 was used for 18 hours. *ldhD* (C), *rpoD* (D). planktonic bacteria change in CFU, Using serial dilutions of Ceftazidime and the correlating cq, with primers for either *ldhD*, or *rpoD* (E). Calibration Curve using eDNA harvested fom bacteria lysed with either heat, or antibiotic indued lysis (F). The results are the average of duplicates, presented as mean ± SD.

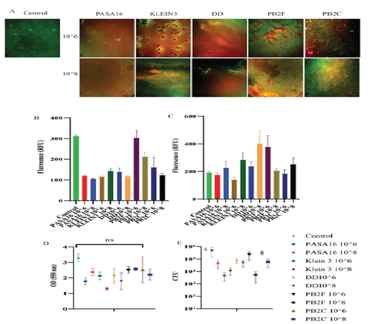

Figure 3. Live Dead staining of pre-grown biofilm treated with different phages at the described concentrations (A), overall GFP fluorescence absorbed from the different treatment groups (B), overall RFP fluorescence absorbed from different treatment groups (C). both Live and Dead stains were able to differentiate between different phages and different concentrations.
Crystal Violet (CV) staining of pre-grown biofilm treated with different phages at the described concentrations. This methods was not able to differntiate between different phage concentrations (D).Sonication and CFU of the described treatment groups, this method was able to differentiate between 3 of 5 phages tested (E).

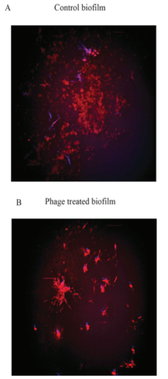

Figure 4. Biolight 680 staining targeted at extracellular matrix. (A-B) Biolight staining (red), DAPI staining (blue), Control (A), KLEIN3 10^8 treated biofilm (B).

CONCLUSION

calScreener – a metabolic methods assessing heat release modification.
pros – sensitive, real time, shows regrow and easy to work with after a short instruction.
cons – expensive, relatively high variance between samples. Current version is anerobic.

qPCR – asses the amount of external DNA (eDNA)
pros- sensitive, utilizes basic lab equipment.
cons- laborious, demands filtration of samples. Not real time and is not able to show regrow. Might be affected from different enzymes present in the environment.

CFU post sonication– assess amount of living bacteria in the biofilm
pros - utilizes basic lab equipment.
cons - not sensitive, not real time

Crystal Violet- assess biomass.
pros - utilizes basic lab equipment.
cons - not sensitive, not real time

Live Dead stain- stains live and dead bacteria with SYTO9, Propidium Iodide accordingly.
pros- sensitive, visual.
cons - demands fluorescence microscopy, not real time.

DAPI, Biolight – Fluorescent stains assessing DNA, Intracellular matrix accordingly.
pros- sensitive, complementary for each other, can be visualized and assessed real time as it is not cytotoxic.
cons- yet to be proven with more data.

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