Mycobacteriophages Exhibit Antibiofilm Activity at High Multiplicities of Infection

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Article

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Abstract

Biofilm formation has been shown to be a very effective survival mechanism used by many bacteria pathogens, including Mycobacterium tuberculosis (Mtb). However, unlike other bacteria, mycobacterial biofilms tend to be very rich in lipids, and this accords them much more resilience than their carbohydrate-based counterparts'. Mycobacteriophage therapy, as an up-and-coming technology, is envisaged to revolutionize the treatment of tuberculosis (TB), particularly involving antibiotic-resistant Mtb. Antibiofilm activity, therefore, is a highly sought-after characteristic of mycobacteriophages intended for therapeutic use. Here we investigated the in-vitro activity of a three-phage cocktail against biofilms of forty-six clinically isolated Mtb using the MBEC biofilm device. We demonstrate that multiplicity of infection and the age of the biofilms are significant determinants of phage antibiofilm activity. Furthermore, based on our host range data, we hypothesize that mycobacteriophages might have a preference for Mtb hosts from pulmonary infection sites compared to those from extrapulmonary sites. If accurate, this finding could have profound implications for both diagnostic and therapeutic applications of mycobacteriophages. Overall, our findings demonstrate the antibiofilm potential of mycobacteriophages and continue to endorse mycobacteriophage therapy as a treatment alternative to our failing antibiotic arsenal. We recommend further investigations to; understand the basis of the observed host preference in mycobacteriophages, evaluate combinatorial therapy of phages and antibiotics, and screen the phages for undesirable genes.

Introduction

Tuberculosis (TB) is still a disease of global concern, over 10 million people developed active TB globally in 2020 and approximately 15% of these succumbed to it. One of the significant challenges to the management of TB is antibiotic resistance. According to the 2021 Global TB report, there were approximately 3.3% new cases of rifampicin-resistant tuberculosis (RR-TB), and 18% from previously treated cases(1). Among others, modified efflux pumps and enzymes that break down the antibiotics are some of the most efficient mechanisms of antibiotic resistance identified in Mtb (2). However, the phenotypic antibiotic resistance, mainly observed in the persistent state, is conceivably analogous to that in biofilm infections of other bacteria such as *Pseudomonas aeruginosa* (3). A biofilm is a multicellular community of microbes, such as bacteria, held together by self-produced extracellular matrices (4). In bacteria of clinical importance biofilms form in response to environmental stressors, such as antibiotics or thiol reductive stress in the case of Mtb, to promote the organism's survival (5). In addition, biofilms are thought to contribute to caseous necrosis and cavity development seen in the lungs during active TB (3). These biofilms act as physical barriers limiting exposure of the embedded infectious bacilli to antibiotics and, to some extent, the host's immune defenses, which often results in resistance to treatment (3, 6). Therefore, effective treatment of bacterial infections should target planktonic bacteria eradication and destroy the associated biofilm structures (7). Different strategies such as, non-invasive means of physical debridement; use of iron-chelating compounds like lactoferrin; and quorum sensing inhibitors, among others, have been used to target biofilms (6). Bacteriophages (or simply phages), viruses that specifically infect bacteria, have also been explored to treat biofilm-associated bacterial infections (8). However, the exact mechanisms through which these phages degrade biofilms are not well understood. Nonetheless, it has been suggested that they propagate in their bacterial host and produce depolymerases that are thought to hydrolyze biofilm

extracellular matrix polymers (9). In the USA, phage preparations, such as, PhagoBurn®, have already been cleared by the Food and Drug Administration (FDA) for emergency treatment of *'biofilm infections'* (10). However, unlike other bacteria, Mtb biofilms are held together by cellulose and mycolic acid-based extracellular matrices, making them more rigid and resilient (11, 12). To this end, it is necessary to evaluate the activity of bacteriophages against Mtb biofilms, which was the aim of this study.

Results

Isolation and characterization of mycobacteriophages

We isolated a total of six mycobacteriophages from all the environmental samples collected, using *M. smegmatis* as the primary host. Only two of these phages, *Mtb01* and *Mtb02*, were isolated by direct spotting on the host lawn. The rest of the phages *Mtb03*, *Mtb05*, *Mtb08*, and *Mtb11*, were isolated after a 24-hour long enrichment in a 48-hour *M. smegmatis* culture. The phages formed plaques of varying morphologies characterised by their size and transparency as described in Table 1.

Table 1: Plaque morphologies and lytic attributes of the mycobacteriophages

		Adsorption time (min)	Latent period (min)	Burst size (pfu/cell)
Phage	**Plaque morphology***	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
Mtb01	Medium, clear	6.7 ± 2.9	45.0 ± 0.0	5.6 ± 2.7
Mtb02	Small, clear	10.0 ± 0.0	43.3 ± 2.9	5.0 ± 1.0
Mtb03	Small clear	10.0 ± 0.0	31.7 ± 2.9	1.4 ± 0.9
Mtb05	Medium, clear	5.0 ± 0.0	28.3 ± 2.9	0.9 ± 0.2
Mtb08	Medium, clear	8.3 ± 2.9	51.7 ± 2.9	6.3±5.1(^10)
Mtb11	Medium, Opaque	13.3 ± 2.9	46.7 ± 2.9	3.0 ± 1.8

*Plaque morphology was determined using high titer phage (>10^8) spots grown on high-density host lawn (>10^7) at 37°C, up to 48 hours in 0.75% top agar. The scale used to define plaque sizes was adopted from Abedon S., 2011. SD- sample standard deviation from three experimental replicates.

Majority of the phages formed clear, medium-sized plaques except for *Mtb11* that formed opaque plaques. The adsorption time ranged between 5-15 minutes, with majority of phages able to infect the host within the first ten minutes of interaction. The latent period on the other hand was between 25 – 45 minutes, and there seemed to be a positive correlation between the latent time and burst sizes of the individual phages, *i.e.,* the burst size appeared to be proportional to latent period. Irrespective, Phage *Mtb11* had a very low burst size given its latent time, a characteristic of lysogenic phages. Unfortunately, lysogeny screening was not performed here. The complete infection cycle generally lasted between 45 to 60 minutes for all the phages, producing on average of one to five new phage particles per host cell except phage *Mtb08*, which averaged a burst size as high as sixty phage particles per host cell; characteristic of highly efficient lytic phages.

Mycobacteriophage host range

Forty-six of the total sixty Mtb target hosts were susceptible to infection by at least one of the mycobacteriophages, (Table 2). The phages *Mtb01* and *Mtb02* had the broadest host ranges both at 77%, followed by *Mtb08* and *Mtb05* at 57% and 55% respectively, while *Mtb03* and *Mtb11* were the only phages with a host range below 50%.

Table 2: Mycobacteriophage host range

	Susceptible hosts				
	Pulmonary isolates		Extra-pulmonary isolates		
Phage	Susceptible hosts (Out of 30)	*Mean E.O.P ± SD	Susceptible hosts (Out of 30)	*Mean E.O.P ± SD	Host range (%)
Mtb01	27	2.9 ± 0.1	19	2.3 ± 0.1	77
Mtb02	27	3.1 ± 0.1	19	2.8 ± 0.0	77
Mtb03	17	0.2 ± 0.2	8	0.2 ± 0.4	42
Mtb05	19	1.0 ± 0.7	14	0.8 ± 0.5	55
Mtb08	22	0.7 ± 0.3	12	0.7 ± 0.3	57
Mtb11	21	0.5 ± 0.5	4	0.2 ± 0.3	42

*Mean plaquing efficiency calculated from only target hosts where the phages formed individual plaques at high dilutions of 10^5 – 10^8.

Given that the spot assay was used to determine the host range, productive infection of the target hosts was indicated by presence of individual plaques at the spot site using very low concentrations of the phage lysate. As a consequence, the EOP was only determined for those phages were individual plaques were formed. Therefore, using EOP as a virulence indicator, based on the results obtained, all the phages were highly virulent on the target host where productive infection was observed.

Host range data also indicated that Mtb isolated from pulmonary infection sites appeared to be more susceptible to mycobacteriophage infection compared to those from the extrapulmonary infection sites. To further explore this hypothesis, we performed a relative risk analysis between these two groups of isolates, as represented in Table 3.

Table 3: *Relative risk analysis for susceptibility to mycobacteriophage among Mtb from different infection sites*

Isolate	Susceptibility to phages		RR (95% CI)	*p-value
	Susceptible	Non-susceptible		
Pulmonary-origin	27	3	1.42 (1.05, 1.91)	0.03
Extra-pulmonary-origin	19	11		

*p-value calculated using Fisher's exact test

Given the relative risk (RR=1.42) in Table 3, Mtb isolates of pulmonary origin were 42% more susceptible to phage infection compared to those from extrapulmonary sites. It is very likely therefore that significant differences exist between these two groups of isolates, particularly at the cell surface where apparatus for phage adsorption are found.

Mtb biofilm formation

Generally, optical density (OD) as an indicator of the biofilm biomass increased with culture period in both the cultures grown in the presence or absence of Tween 80 as represented in Figure 1.

At one-week old, there was no difference between ODs of biofilms grown in the presence or absence of the biofilm inhibitor, Tween 80. On the other hand, at two-weeks old, ODs of biofilms grown in absence of Tween 80 were much higher than those grown in the presence of Tween 80 as represented in Figure 1. This therefore suggests that immature and mature stages of biofilm formation were successfully established at one and two weeks of growth, respectively.

We also compared biofilm formation among the Mtb isolates to determine whether any patterns existed relative to the infection sites as represented in Figure 2.

Generally, the observed patterns of biofilm formation were unique to the individual isolates and there was no apparent difference to distinguish between biofilm formation in pulmonary and extrapulmonary isolates. Nonetheless, we performed a one-way ANOVA (Table 4) and concluded that there was no significant difference in biofilm formation between the two groups of isolates. All isolates except from sputum and CSF, displayed a progressive patter of biofilm formation where by the biomass increased nearly two fold from the immature to the mature stages of growth. Blood and lymph node aspirate isolates not only displayed the same pattern of biofilm formation but also had similar biomass across the entire growth period. Interestingly, multidrug resistant sputum isolates *i.e.,* Sputum (MDR), did not share the same pattern with the other sputum isolates, and in addition formed the heaviest biofilms at both immature and mature stages. Owing to this observation, it is very reasonable to think that the MDR-trait had a significant bearing on biofilm formation.

Table 4: *Single factor ANOVA for mean optical densities of biofilms between Pulmonary and Extra-pulmonary Mtb isolates*

SUMMARY						
Groups	**Count**	**Sum**	**Average**	**Variance**		
Pulmonary	27	6.22555955	0.23057628	0.07113797		
Extrapulmonary	19	4.78172361	0.25166966	0.06899074		
ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	**p-value**	**F crit**
Between Groups	0.00496195	1	0.00496195	0.07062308	0.791673	4.06170646
Within Groups	3.09142051	44	0.07025956			
Total	3.09638245	45				

Mycobacteriophage anti-biofilm activity

To varying extents, the three-phage cocktail comprising *Mtb01*, *Mtb02* and *Mtb08,* was able to both inhibit and disrupt biofilm formation as given by the percentage reduction in biofilm mass against one and two weeks old biofilms, respectively. The phages displayed unique patterns of antibiofilm activity against individual Mtb isolates as represented in Figure 3.

Antibiofilm activity was highest against pleural fluid isolates, approaching 60% reduction in one week old biofilms. On the other hand, the lowest antibiofilm activity was against blood isolates, averaging about 20% for both the mature and immature biofilms. This was interesting given that blood and lymph node aspirate isolates displayed the same pattern of biofilm formation (Fig. 2), yet the former now appeared to form more resilient biofilms. Generally, antibiofilm activity increased with MOI, on average achieving peak activity at MOI of 100. Contrary to this, antibiofilm activity against sputum (MDR) isolates was instead maintained at an average of 40% reduction in biofilm mass across the different MOIs for both mature and immature biofilms.

We further explore the relationship between MOI and antibiofilm activity by performing a linear regression to evaluate the direct effect of the MOI on antibiofilm activity (Figure 4).

As represented in Figure 4, antibiofilm activity was directly proportional to MOI; where every unit increase in the MOI coincided with a 7.5% or 4.0% reduction in biofilm mass against immature or mature biofilms respectively. Considering the regression model generated, 90% and 80% of the observed reduction in biofilm mass against immature and mature biofilms respectively, was attributable to an increase in MOI. The differences in these observations also suggested that biofilm age was an important determinant of phage antibiofilm activity.

Discussion

To date, over 700 mycobacteriophages have been isolated mainly from environmental sources using *M. smegmatis* as a primary host(18). Majority of these phages however, target NTMs and only a handful of them can ably infect Mtb(19). All six mycobacteriophages isolated in our study had lytic activity against Mtb, with four of them exhibiting host ranges above 50%. Studies looking at the genetic diversity have associated the potential of mycobacteriophages to ably infect Mtb with membership to particular genome clusters (19). Unfortunately for us, no whole genome sequence data was available to determine which genome clusters the phages belonged to. However, due to the fact that these phages were isolated from sewers draining one of the largest prisons in Uganda, we hypothesise that this too could have influenced the abundance of Mtb specific phages in this kind of environment. A recent study investigating the fate of Mtb in the environment highlighted the occurrence of viable Mtb cells in environmental sources including sewage(20). Given that prisons are considered reservoirs of tuberculosis in the environment (21), it is quite plausible that effluent water from such places could have an unusually high numbers of viable Mtb cells, thereby influencing the abundance of Mtb-specific phages in such samples.

Despite their high host range, phages exhibited a preference for pulmonary Mtb isolates compared to the extra pulmonary ones. Generally, phages can infect members of the same species at different efficiencies, but total selectivity within a species is not a common occurrence(22). Swift *et al.,* however, demonstrated that the mycobacteriophage D29 preferentially infected mycobacteria grown in the presence of oxygen to that grown in oxygen deprived conditions; it is thought that under different metabolic states, there are changes in the cell's biology that could render the bacterium tolerant to phage infection(23). On the other hand, the abundance of 'phage receptors' on the bacteria cell wall could also explain why pulmonary isolates appeared to be more susceptible to phages compared to the extra pulmonary ones. Pathophysiological studies have demonstrated how the lung environment (immune cells plus the lung microbiome), can influence the nature and number of receptors on Mtb (24), thereby inadvertently affecting their susceptibility to phages *i.e.*, causing them to be more or less susceptible to phage infection. Clearly, host preference in mycobacteriophages is a huge research gap with potentially significant implications for both diagnostic and therapeutic phage applications.

However, the observed differences between pulmonary and extra pulmonary Mtb isolates seemed to be limited to only susceptibility to phage infection. Biofilm formation on the other hand was unique to the individual isolates, with no apparent distinction between the two broad categories. Nevertheless, it was intriguing to note that MDR-TB isolates from sputum not only formed heavier biofilms than other sputum isolates, but altogether formed the heaviest biofilms. Based on this observation alone, an ardent association can easily be made between the MDR trait and biofilm formation. Indeed majority of *in-vitro* experiments have consistently described drug tolerant Mtb as perpetual residents in biofilms (3, 25). However, there is currently no evidence to support this claim. As a matter of fact, none of the genes involved in development of resistance towards anti-TB drugs, including the new and repurposed ones, are important in biofilm formation(2, 12). Reductive stress however, was shown to promote biofilm formation, as an *in-vitro* coping mechanism in Mtb (5). Overall, there is very limited information to explain the differences in biofilm formation observed here.

Phages exhibited both inhibitory and disruptive antibiofilm activity, albeit to different extents against the Mtb isolates. Generally, the combined activity was higher in immature biofilms compared to the mature ones. This

was unsurprising considering the differences in metabolic states and arrangement of cells between mature and immature biofilms. In mature biofilms, the outermost layers comprise cells that are in a constant non-reproductive state and tightly packed, leaving very few spaces through which phages can percolate to access the deeper layers which have the actively dividing cells (25). A similar pattern of phage activity has been observed against *M. smegmatis* biofilms (26), implying that biofilm age is a major determinant of phage antibiofilm activity.

Phage MOI was also shown to affect antibiofilm activity. At least 70% of the observed increase in antibiofilm activity was attributable to an increase in the MOI. Theoretically, high MOIs increase the number of phages per unit area of the biofilm, which increases the likelihood that the phages will access the deeper layers where they can infect metabolically active biofilm cells (28); essentially destroying the biofilm from the inside-out. Likewise, phages with high burst sizes have consistently displayed higher anti-biofilm activity compared to those with lower burst sizes (26). This however, is only true if the mechanism of antibiofilm activity is via productive infection of the metabolically active biofilm-cells(27). On the other hand, if phages are destroying biofilms via 'production' of lysins or depolymerases (28), we would expect the antibiofilm activity to follow a pattern similar to that observed here against MDR-TB isolates from sputum

From our observations, MOI and age of the biofilm were major determinants of phage antibiofilm activity. In *in-vivo* settings however, it is very difficult to estimate these parameters, not to mention that there are other key players such as the host immune cells and the microbiome, all of which can profoundly affect phage activity. Moreover, biofilms can be very complex structures, sometimes involving more than one species, which makes them impossible to dislodge with a species-specific phage cocktail. Therefore, to realise the therapeutic potential of phages, human intervention is necessary in order to manoeuvre these complexities and/or mitigate any resistance by the pathogen. One way of doing this for example, could be treating biofilms using a combination of phages/phage-derived enzymes and antibiotics. Otherwise, phages and bacteria have co-evolved for millions of years, and if left in their natural state, their interactions, are essentially a cat-and-mouse game.

Methods

Study design, site, and setting

This was a laboratory-based experimental study carried out at the Microbiology research laboratory at the College of Veterinary Medicine, Animal Resources and Biosecurity (CoVAB) and the College of American Pathologists ISO15189-Accredited Mycobacteriology Biosafety level III (BSL-3) Laboratory at the College of Health Sciences (CHS), Makerere University. Soil and sewage samples for mycobacteriophage isolation were collected along the Nakivubo sewage channel in Luzira-Kampala.

Study entities and sampling

Soil and sewage samples for phage isolation were collected at different points along the Nakivubo sewage channel in Kampala. The mycobacterial isolates used were obtained from stock cultures at the

Mycobacteriology laboratory; *Mycobacterium smegmatis* mc^2164 was used for initial isolation and propagation of mycobacteriophages. Archived Mtb clinical isolates were used to determine the mycobacteriophage host range and anti-biofilm activity. These isolates were selected to represent pulmonary and extra-pulmonary infection sites. For the pulmonary sites, isolates originally obtained from sputum or the pleural fluid were used, while for the extra-pulmonary sites, isolates originally obtained from blood, lymph nodes, and Cerebral spinal fluid (CSF), were used. In total, sixty Mtb isolates were used, ten for each infection site, except 'sputum', n=20.

Preparation of phage-host stock cultures

M. smegmatis mc^2164 was used for initial isolation as well as propagation of the mycobacteriophages. The bacterium was grown on 7H10 (Sigma-Aldrich) agar at 37°C for 48-hours to form pure colonies. One discrete colony was then cultured in 7H9 broth (Sigma-Aldrich) supplemented with 10% customized Albumin-Dextrose-Sodium chloride solution (ADS). The ADS was composed of 5.0g Bovine Serum Albumin (Sigma-Aldrich™), 1.0g Dextrose (Sigma-Aldrich™), 0.425g NaCl in 50ml of pure water. Stock cultures of the bacterium were maintained at 1.5×10^8CFU/ml at 4°C.

Isolation of mycobacteriophages

Mycobacteriophages were isolated by both the direct spotting method as well as enrichment method described by Jassim and Limoges (13) with some modifications. Briefly, 50ml of sewage were centrifuged at 10,000 × g for 10 minutes to remove particulates. On the other hand, 10g of soil samples, on the other hand, were suspended in 50ml of phage extraction buffer comprising 50mM Tris-HCL (Merck™), 150mM NaCl, 2mM $CaCl_2$, and 10mM $MgSO_4.7H_2O$. After centrifugation, the supernatant was filtered through 0.45µm low protein binding membrane filters (Corning™) to obtain a filtrate with possible active phage. A portion of the filtrate was maintained at 4°C until spotting, and the rest was enriched by adding an equal volume of double strength 7H9 broth supplemented with 100µL of a 48-hour culture of *M. smegmatis*. The culture was incubated at 37°C with gentle mixing at 110 RPM for 48 hours. After incubation, the culture was centrifuged at 10,000 × g for 10 minutes and filtered through a 0.45µm membrane filter to obtain lysate with active phage. A spot lysis assay described by Jassim and Limoges was used, with some modifications, to test for the presence of phage in both the enriched and non-enriched filtrate. Briefly, a host bacterial lawn was made by casting 3ml of molten top agar (7H9 broth with 0.75% bacteriological agar maintained at 46°C) mixed with 100µl of a 48-hour culture of *M. smegmatis* mc^2164 onto 7H10 agar supplemented with 2mM $CaCl_2$. The agar was allowed 30 minutes to set before spotting ten microliters of the filtrate onto the bacterial lawns. The spot was left to dry before incubation at 37°C for 48 hours. Detection of phage was based on the visual appearance of either clear or semi-clear (turbid) lysis zones.

Purification of mycobacteriophages

As described by Jassim and Limoges, the double agar overlay technique was used to purify the phages through multiple rounds of plaque clean-up. Briefly, a discrete plaque formed following the spot test was

emulsified in one milliliter of cold phage extraction buffer (0.01M Tris HCL pH 7.5, 0.01M MgSO4, 0.1M NaCl), and incubated at 4°C for one hour. The solution was cleaned by passing it through a 0.45mm syringe filter. The filtrate was serially diluted nine times in a 10-fold pattern using 7H9 broth. Ten microliters of each dilution were individually mixed with 200mL of a 48-hour culture of *M. smegmatis*. The phage-bacteria mixture was incubated at 37°C for thirty minutes to facilitate phage absorption. After incubation, the culture was mixed with 3ml of top agar at 46°C and over-laid onto 7H10-agar plates. The plates were incubated until plaques were observed. This process was repeated until morphologically uniform plaques were obtained on at least two consecutive times. The purified phages were multiplied to titers between 10^8 and 10^{10}, and maintained in three 1ml stocks at 4°C.

Characterization of mycobacteriophages

Mycobacteriophages were characterized by their plaque morphology, adsorption time, burst time, and size. Pure phage plaques were obtained by performing the double agar overlay techniques as described earlier. Morphologies of pure phage plaques were characterized according to their size and clarity. A one-step growth experiment as described by Middelboe et al., (14) was performed with some modifications to determine the adsorption time, burst time, and size. Briefly, 50ml of $1.5X10^8$ cfu/ml *M. smegmatis* culture was mixed with pure phage of known titer to give a multiplicity of infection (MOI) of 0.01. One milliliter aliquots were withdrawn from the phage-bacteria mixture at five-minute intervals for up to sixty minutes. The aliquots were centrifuged at 6,000g for 10 minutes. The supernatant was poured out, and the pellet was washed twice in phosphate-buffered saline (PBS) in order to get rid of all unabsorbed phages. The pellet was resuspended in one milliliter of 7H9 broth, serially diluted and the plaque-forming units determined using the plaque assay. Adsorption time was determined by the lowest time interval required to produce the highest phage progeny.

Culture of Mtb

Mtb study isolates were selected from the BSL3 laboratory and the H3Africa biorepository of the School of Biomedical Sciences, Makerere University. The isolates were rapidly thawed by incubating them at 37°C for one hour. After thawing, 100µL of each isolate was spread onto 7H11 agar (Sigma-Aldrich™) supplemented with a selector tab (Kirchner Selectatab®). The cultures were grown for up to twenty-one days at 37°C. Colonies characteristic of Mtb, *i.e.*, large, dull-grey colored, rough-textured and tend to adhere to the agar; were aseptically transferred into 10ml of 7H9 broth and grown at 37°C for up to seven days. The MPT64 rapid diagnostic test (Standard Diagnostics Incorporated) was used to confirm whether the cultured isolates belonged to the Mtb Complex (MTBC). The cultures were then maintained in 10ml of 7H9 broth at 37°C until required.

Determination of mycobacteriophage-host range

Mycobacteriophage host range was determined using the spot test and relative efficiency of plaquing (EOP) assay, which were performed sequentially. Briefly, 10µL of high titer lysate for each bacteriophage was spotted onto individual lawns for all the sixty Mtb isolates. The spot was left to dry, and thereafter the culture

was incubated at 37°C for up to two weeks. The plates were sealed using micropore tape (Millipore™) to minimize dehydration of the agar. After incubation, the cultures were observed for zones of lysis in the areas spotted with the lysate. Lysis zones were scored using a system adopted from Kutter *et al*., (15), i.e. 0, +1, +2, +3 and +4 according to the size and clarity of the plaques. The relative EOP was only performed for phage-host interactions where lysis zones of score +1 and above were observed. The relative EOP was performed as described by Kutter *et al*., (15) with some modifications. Briefly, several serial dilutions of high titer phage were made using phage extraction buffer. For each mycobacteriophage, two agar overlay assays were performed; with one having *M. smegmatis* as the host, while the other had the target Mtb isolate. In both cases, the hosts were used at the same concentrations. The plates were then incubated at 37°C for up to two weeks. Plates with plaques ranging from 30-300 were selected for enumeration and calculation of the plaque-forming units per milliliter (PFU/ml) using the formula below.

$$\text{PFU/ml} = \frac{\text{number of plaques counted} \times \text{dilution factor for the plate counted}}{\text{Volume of phage inoculum added}}$$

The relative EOP was calculated using the formula below:

$$\text{Relative EOP} = \frac{\text{average PFU on target Mtb isolate}}{\text{average PFU on } M.smegmatis}$$

Phages with EOP values of 0·5–1 were ranked as 'highly efficient'; 0·2–0·5 as 'medium efficiency'; 0·001–0·2 as 'low efficiency'; and 0·0 was considered as not effective against the target strain (16).

Establishing Mtb biofilms

The Minimum Biofilm Eradication Concentration (MBEC) assay plate, also known as 'MBEC plate,' was used to grow the biofilms of Mtb. The MBEC plate is a 96-well plate consisting of a cover with pegs that fit into each of the ninety-six wells that make up the base of the plate. The conical shape of the pegs and the constant rotation of the broth inside the wells around the pegs promotes biofilm formation far much more than the conventional microtiter plate method. To inoculate the MBEC plate, each of the ninety-six wells was filled with 150µL of either sterile water or 7H9 broth. A bacterial inoculum of approximately 1.5×10^8 cells per milliliter was prepared by diluting a seven-day-old culture of a particular Mtb isolate in Dulbecco's phosphate-buffered saline (Merck™, Germany). Freshly prepared McFarland standard No. 0.5 (0.05ml of 1% Barium chloride and 9.95ml of 1% sulfuric acid) was used to standardize the turbidity of the inoculum. Fifteen microliters of the inoculum were aseptically transferred into designated wells. In order to reduce the rate of evaporation as well as prevent cross-contamination, wells on the perimeter of the plate and those separating individual isolates were filled with sterile water and 7H9 broth, respectively. The plates were then covered such that the pegs only fit into their designated wells. The plates were sealed with micro-pore tape to reduce the rate of evaporation. The plates were incubated in a humidity-controlled orbital shaker (New Brunswick Scientific™) at 37°C, 134 RPM. Fresh media refills of 50µL 7H9 broth were done every three days in

each of the inoculated wells taking care not to disturb the biofilms on the pegs. To establish active (immature) and standing (mature) biofilms, these cultures were incubated for seven and fourteen days, respectively. A negative control plate having 7H9 broth supplemented with 0.1% Tween 80 was inoculated and set alongside the test plate. The amount of biofilm formed on the pegs was quantified using the Crystal Violet (CV) staining technique previously described by Knezevic (17). Briefly, MBEC plate pegs were carefully withdrawn from the wells and then gently washed in Dulbecco's PBS, pH 7.0 (Sigma-Aldrich, USA), to remove the non-adherent cells. The pegs were then covered in absolute methanol for 15 minutes to fix the biofilms. After drying, the pegs were stained with a 0.5% w/v solution of CV (Conda®) for 30 minutes before rinsing twice in excess PBS. The biofilm-associated dye was eluted using a 33% w/v solution of glacial acetic acid (BDH®) for 20 minutes. The intensity of the eluted dye was measured as optical density (OD) using a microtiter plate reader (MR-96 CLINDIAG™) at a wavelength of 600nm. The higher the observed optical density, the greater the amount of biofilm formed.

Determining anti-biofilm activity of mycobacteriophages

Mycobacteriophages' inhibitory and disruptive anti-biofilm activity was determined by infecting biofilms with the phages at week-1 and week-2 of growth, respectively. Bacteriophages were added in MOIs of 100, 10, 1, 0.1, and 0.01. Fifty micro-liters of the phage cocktail were added to appropriate wells, and the plate was incubated for seven more days under the same conditions. A control plate without phage was grown alongside the test plates for each Mtb isolate. After incubation, the pegs were removed and stained as described above. Mycobacteriophage anti-biofilm activity was expressed a percentage reduction in biofilm given by the formula below:

$$\%\ \text{biofilm reduction} = \frac{\text{OD of non} - \text{phage treated biofilms} - \text{OD of phage treated biofilms}}{\text{OD of non} - \text{phage treated biofilms}} \times 100$$

The inhibitory and disruptive activity was given by the percentage reduction calculated for active and mature biofilms, respectively.

Data analysis

Data was entered and cleaned in Microsoft Excel Version 16, and analysed using JMP Pro Statistical Software. A One-Way Analysis of Variance (ANOVA) was performed to define any differences in observed optical densities between the phage treated and the non-phage treated biofilms. Risk ratios were used to compare phage susceptibilities between isolates from the pulmonary and extra pulmonary infection sites. A simple linear regression analysis was used to determine the relationship between antibiofilm activity and MOI of the phages.

Ethical consideration

This study was approved by the School of Biomedical Sciences Research Ethics Committee (SBS REC), under REC number SBS-754.

Conclusion

In light of the current challenges to the treatment of tuberculosis due to the advent of MDR, XDR, and TDR isolates of Mtb, therapeutic alternatives, including the use of bacteriophages, have been suggested. This study has shown the potential of mycobacteriophages to both inhibit and disrupt biofilm formation, a contributor to antibiotic tolerance, in Mtb. However, as is the case with most current efforts in phage therapy, additional research is crucial to replicate these findings *in-vivo*. We recommend that future studies focus on understanding; the mechanism(s) underlying host preference in mycobacteriophages, structural and pathophysiological differences of Mtb isolates in disseminated tuberculosis, and the combined therapeutic effect of phages and antibiotics against tuberculosis.

Declarations

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AUTHOR CONTRIBUTIONS

DK- conducted laboratory experiments and compiled the manuscript

JLN-supervised the laboratory work and reviewed the manuscript

BA- supervised the laboratory work and reviewed the manuscript

JS- conducted laboratory experiments and reviewed the manuscript

WS- conceptualized the idea, supervised the laboratory work, and reviewed the manuscript

COMPETING INTERESTS

All authors declare no competing interests

MATERIALS AND CORRESPONDENCE

All correspondences and requests for material used in this study should be made to WS

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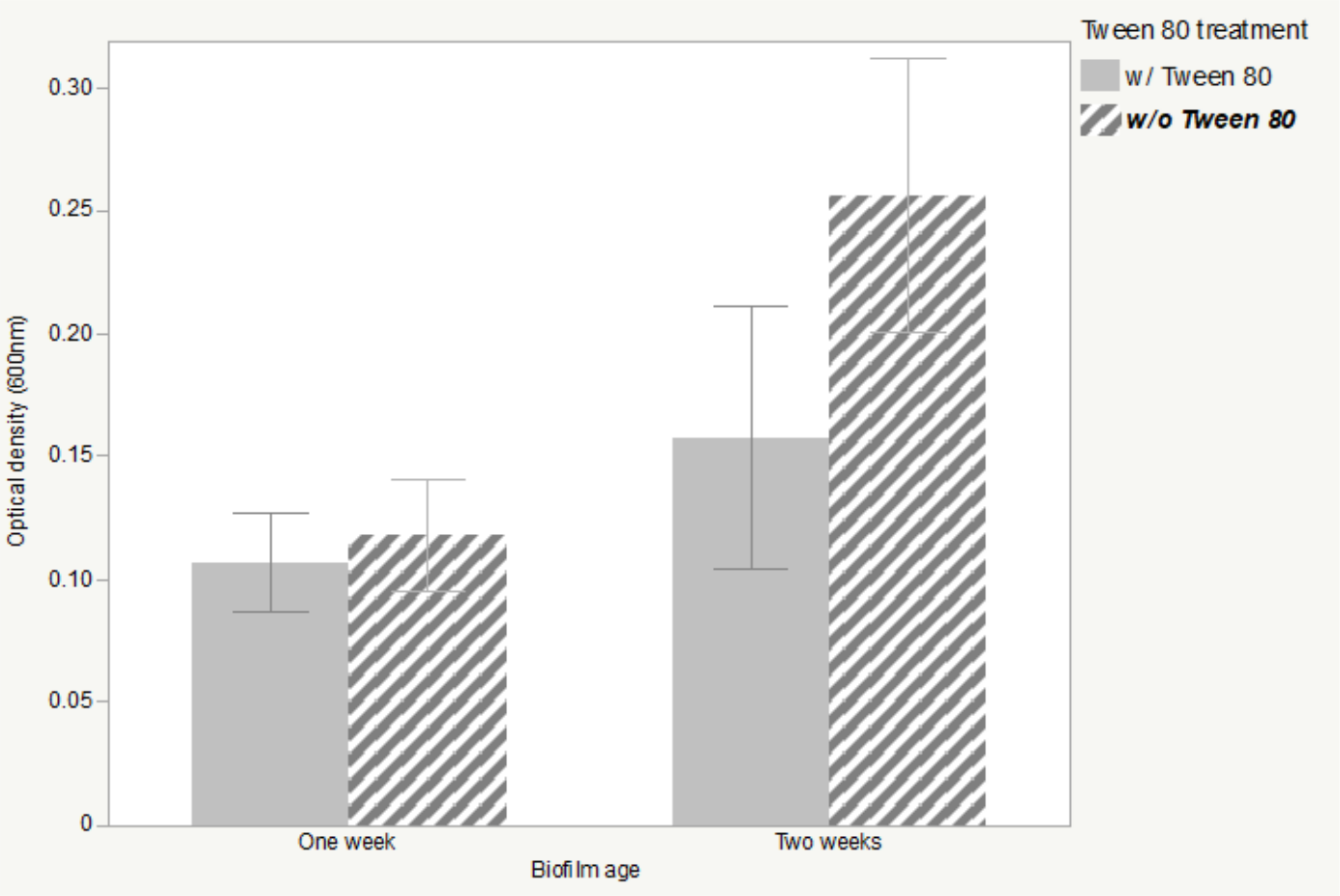
Figures



The error bars represent the standard deviation of the mean OD observed across three experimental replicates. Mtb isolates, n=46

Figure 1

Optical densities of Mtb biofilms cultured in the presence or absence of Tween 80

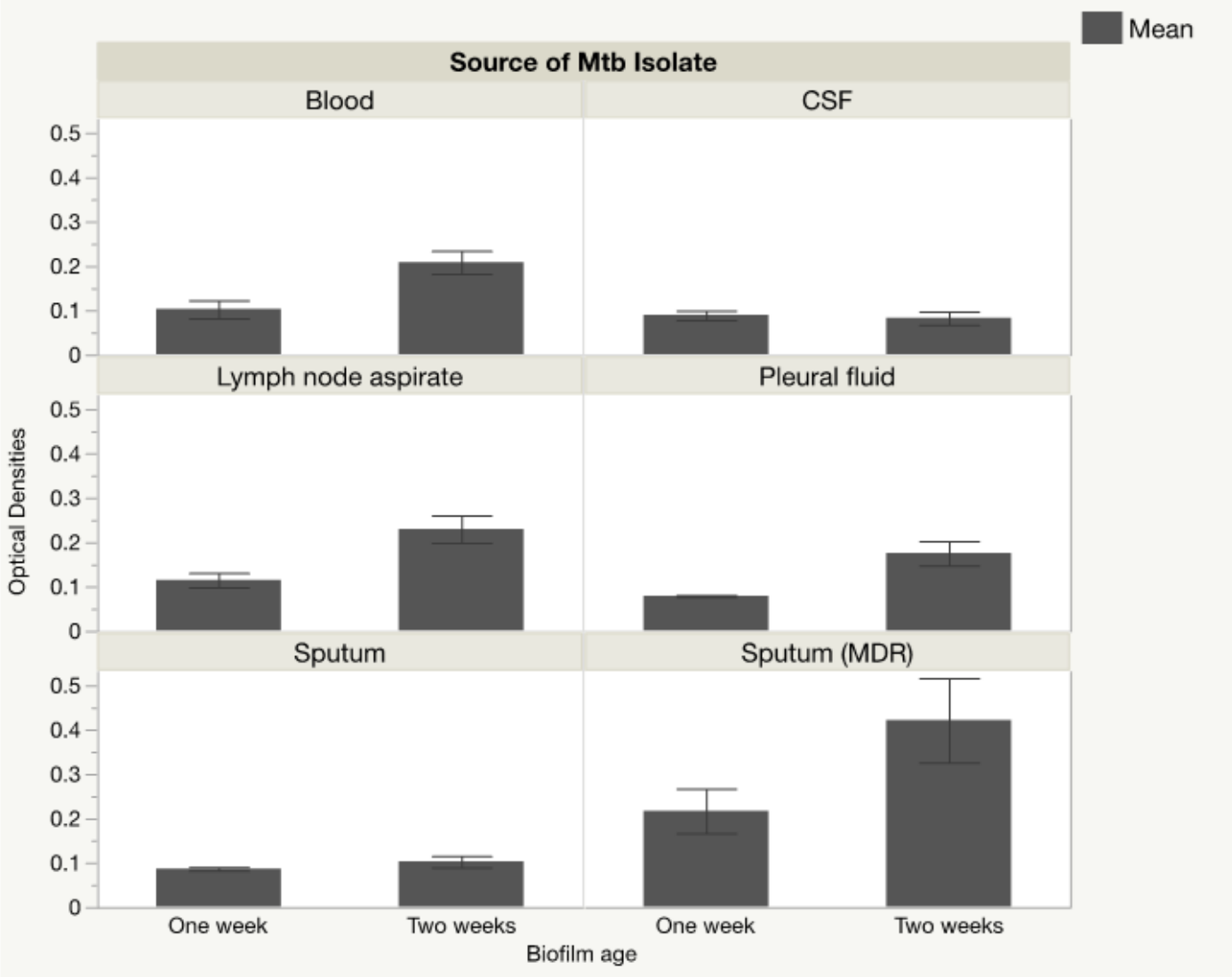


Figure 2

Comparison of biofilm formation among Mtb from different infection sites

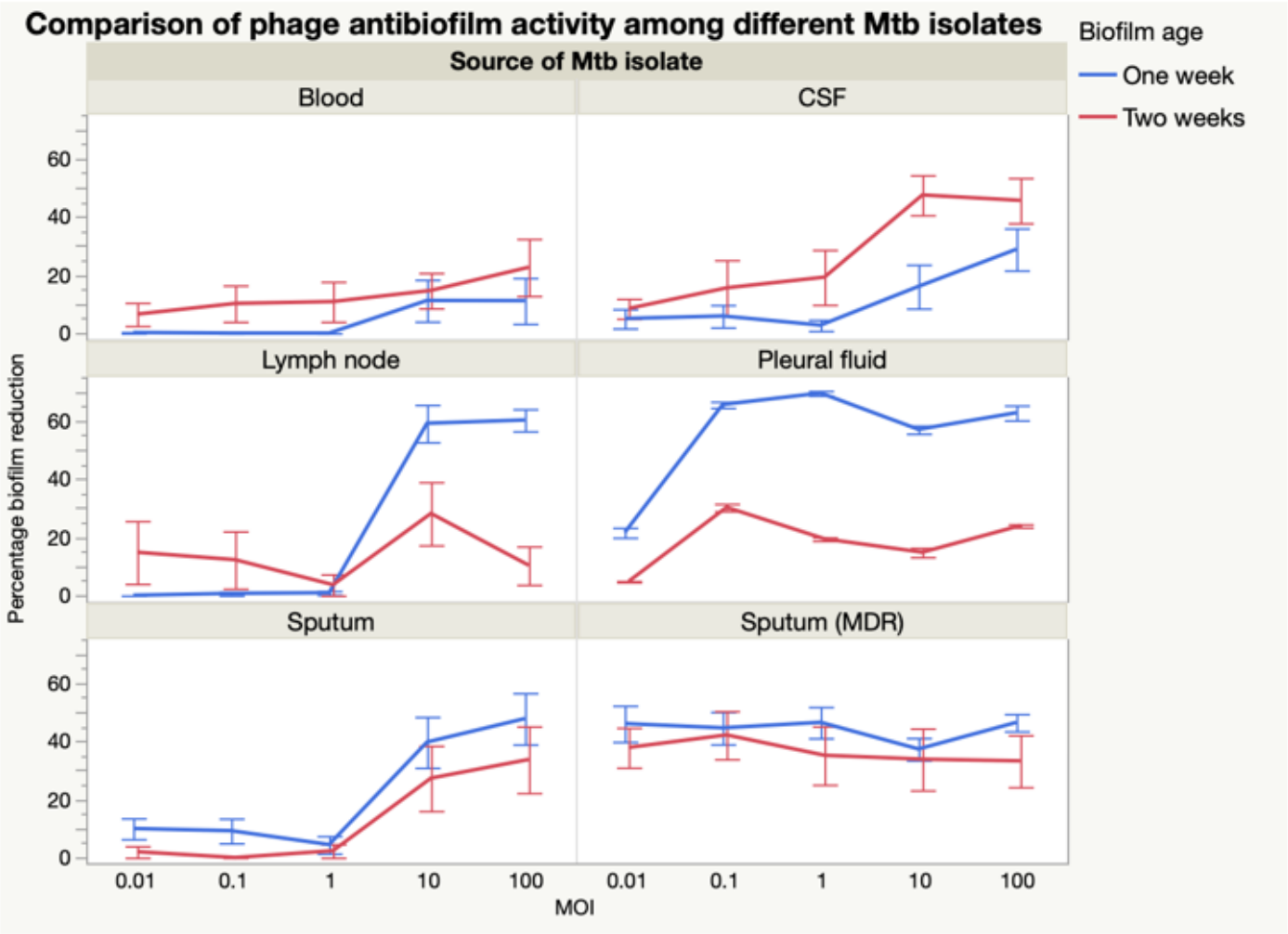


Figure 3

Phage antibiofilm activity against mature and immature Mtb biofilms

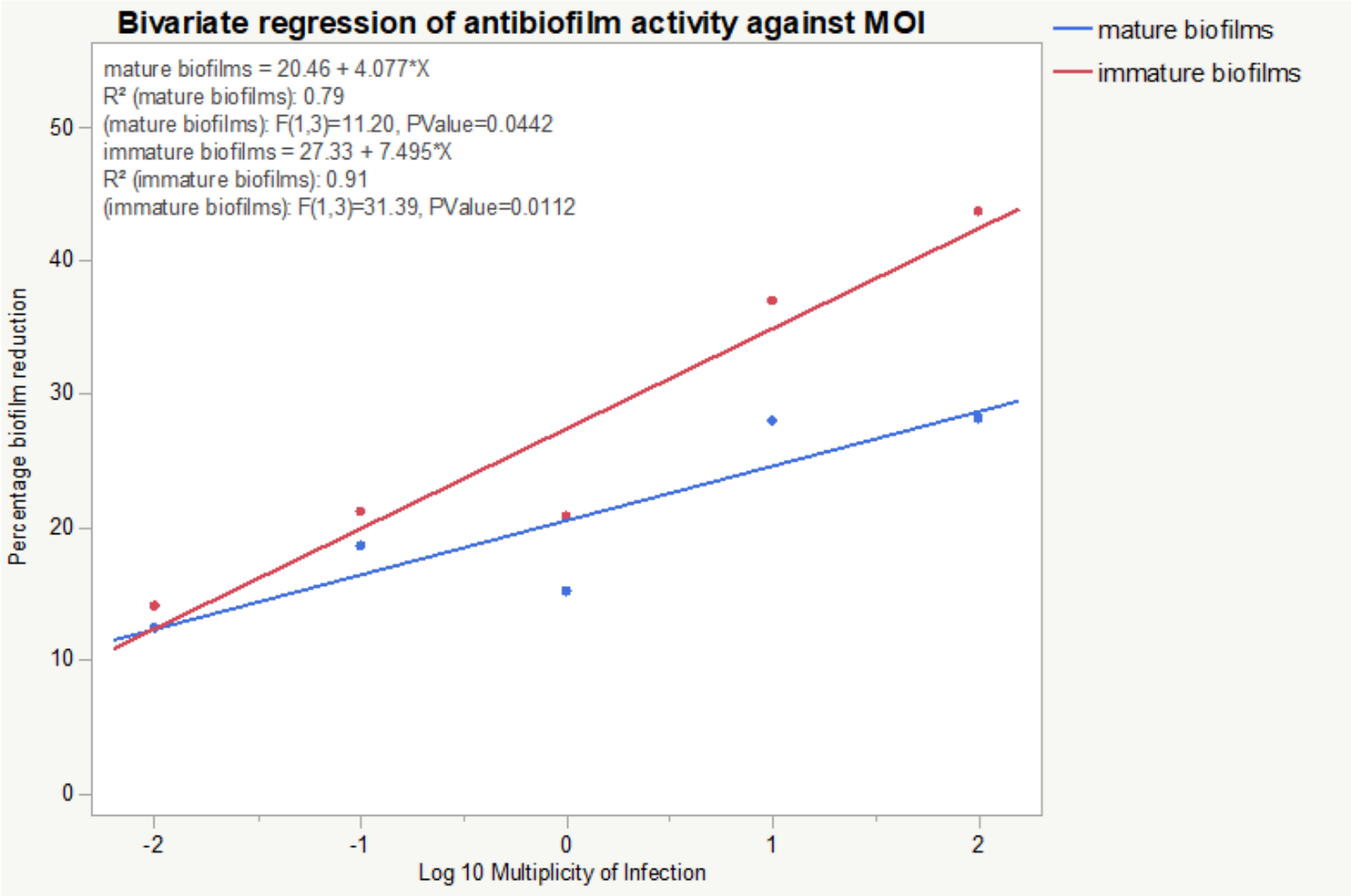


Figure 4

Linear relationship between phage antibiofilm activity and MOI