Original Article

Novel methods to design wild bacteriophages into highly lytic and therapeutic bacteriophages to extensively drug-resistant Mycobacterium tuberculosis

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Abstract:

Background: The emergence of multiple drug resistant (MDR) Mycobacterium tuberculosis (M.TB) and extensive drug resistant (XDR) M.TB lay huge burden on TB endemic countries such as Iraq.

Objectives: Bacteriophage (phage) therapy can be used as alternative approach to tackle this problem.

Patients and methods: Forty isolates of M.TB were cultured from TB-positive sputum specimens with three ATCC strains. Phage passaging and biokinetic based techniques were used to optimize wild anti-M. TB phages. Three chemical, non genetic- designing techniques, tween-80, mycobacterial lysis buffer, and xyelen, were used to change the specificity of wild anti-TB phages towards phage-resistant target M.TB.

Results: Five wild anti-M.TB phages were isolated and optimized. The optimization techniques were successful in enhancing plaques size, clarity, burst size, and infective ratio. Chemical designing techniques succeeded to reorient specificity of 6 phages to new host bacteria.

Conclusions: phage designing opens door wide for endless future applications of phage-based therapy, biocontrol and diagnosis.

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Keywords: bacteriophage, Mycobacterium tuberculosis, phage therapy, phage cocktail, phage designing, MDR bacteria.

Introduction:

Multi-drug-resistant Mycobacteria including Mycobacterium Tuberculosis (MDR M.TB) are resistant to at least two antibiotics (1-3). For M. TB, two anti Tuberculosis medicines, namely rifampicin and isoniazid have become ineffective. These medicines have long been used as first line drugs (4). MDR TB is defined as that bacteria should be at least resistant to both isoniazid and rifampin, which are the most effective anti-TB drugs. Another modality of resistant TB is the extensively drug resistant (XDR TB); it is an aggressive form of MDR M.TB which is not susceptible to isoniazid and rifampin, along with any fluoroquinolone and at least one of three injectable second-line drugs, namely amikacin, kanamycin, or capreomycin (5).

MDR TB bacteria were addressed by the WHO as global serious health problems. In general, conventional antibiotics fail in the race versus resistance emergence, however there is another weapon has long been underestimated but now there is a re-entry into the field as one of the most successful anti-bacterial alternatives, namely bacteriophages (6, 7). In this study, bacteriophages were used as the ultimate bacterial predator and/or indicator for therapy, biocontrol and diagnosis purposes to M. TB.

This study is directed towards one of the most challenging health issues all over the world, namely the bacterial infections that are resistant to all or most of antibiotics, particularly multiple drug resistant MDR TB infections and the other problem is the time consuming and expensive diagnostic essays used for rapid and accurate TB diagnosis. TB infection is remerging in the world including poor areas and poor population sectors (8-11). It is well-known that TB diagnosis and therapy is costly. Moreover, TB therapy lasts for years ending sometimes with no encouraging results. The cost of using long term 'atypical TB therapy regimen' is highly expensive for average income people (1). In addition, the process of developing resistance against the conventional antibiotics has been progressing much faster than discovering new antibiotics (4).

In the current study, bacteriophages were collected from the wild, optimized, and designed by novel phage design and breeding techniques to create a set of anti-TB bacteriophages that capable for killing the bacteria by using both systemic and topical routes of administration. Furthermore, phage lysins enzymes will be intended to be separated from the TB- specific phages. This will allow a breakthrough chance of finding a chemical substance capable of lysing the infective mycobacterium TB.

Patients and Methods

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Isolates of M. TB: Forty isolates of already diagnosed and stocked as XDR M. TB were thankfully granted from Bacterial Laboratory, Serdang Hospital, Malaysia. Phage isolation, optimization and designing were conducted in the laboratories of University Putra Malaysia, Institute of Bioscience. Since this project is self-funded and a single-authored study, it was conducted on three phases, first phase during the period from January 2011 to July 2011 for phage isolation and optimization, second phase from 1st July-30th August 2013 for continuation of phage optimization and starting chemical designing and from 3rd July-4th September 2014 for continuing chemical designing of phages.

Propagation of M.TB isolates: Stock samples of M.TB were prepared using N-acetyl L-cysteine sodium hydroxide method by inoculating on slants of 7% sheep blood agar rather than Lowenstein Jhonson (LJ) medium. Samples were incubated at 37 C for every day observation looking for visible growth (12). Blood media for M.TB were prepared as follows: 20 ml of sheep blood were added to 4 ml of sodium citrate (3.8%) solution. About 100 ml of DW with 4g of Columbia blood agar base (Pharma Ltd. UK) and 0.5 ml of crystal violet solution (0.02%) was boiled in order to dissolve media well. Afterwards, 7 ml of citrated sheep blood were dissolved in it. To minimize contamination, 90 ul of nystatin and 50 ul of antibiotic solutions were added (88.8 mg Polymixin-B, 5 mg trimethoprim and 20 mg nalidixic acid) (12). For phage propagation, the same sheep blood media was used except for agar in order to obtain sheep blood broth medium able to support both Mycobacteria and bacteriophages as well. The reason behind using sheep blood media rather than LJ medium is that blood media allow more rapid growth of M.TB than LJ. The mean time to detect macroscopic colonies in blood agar medium was 2 weeks only. Three reference strains were used, namely ATCC 27294, ATCC 25177, and ATCC 25618. The isolation and processing of M.TB isolates as well as reference strains were done in biosafety level 3 cabinets. Since no lab animals or humans were dealt in this study, ethical approval was straight forward given by University Putra Malaysia.

Isolation of wild phages against M. TB: The method used for isolating anti-TB phages from crude soil specimens was principally based on the method used by our team before on different bacteria bith with modifications suiting TB bacteria (13). Fifty gram of soil/sewage specimens were collected in a sterile 100ml tube. One to three grams of the collected specimen were mixed in 90 ml of 7% sheep blood broth with 30 sec vortexing. Then, 1ml of 2-weeks blood broth cultures of each one of the 40 TB isolates along with standard, or reference, strains were incubated at 37ºC. After 3days, 10 ml of the mixture were centrifuged 5000xg at room temperature for 5 min. Afterwards, 1 ml chloroform (Sigma, USA) was added to the supernatant in a sterile 15 ml tube with 5 min gentle shaking and then incubated on crushed ice for 5 min. A cloudy solution resulted because of the digestion of bacterial proteins by chloroform. After centrifuging 5000xg for 5 min at room temperature, the upper aqueous layer was collected and stored at 4ºC (13).

Testing for the presence of wild phages: This testing is based on creating bacterial lawns from the isolated bacteria and reference strains. Lawns were produced by pouring 1 ml of 2-weeks culture blood broths on blood agar plates till dry. Ten µl of the possible phage suspension were spotted on the bacterial lawns and then incubated at 37 C and lysis spots were observed after 10 days. The assessment of positive results was according to the presence of lysis spots and their level of clarity, whether clear or semi-clear (turbid); on the other hand, negative results were assessed by the absence of lysis spots (13).

Preparation of phage stock: Lysis spots, if any, were separated from the lawn and put in 0.5 ml Lambda buffer for 20 min with slow rotating shaking. A 1:10 chloroform: lysate ratio was used along with gentle shaking for 5-10 min for eluting phages from the separated agar of the lysis spot as well as for disintegrating bacterial host cells. Afterwards, the chloroform-treated mixture was incubated in ice for 5 min; then, it was centrifuged at 5000xg for 15 min at room temperature. The resulted supernatant was the phage suspension which was withdrawn into 1.5 ml sterile tubes (14).

Optimization of the phages lytic characteristics: *Plaque-based optimization:*

The isolates of wild lytic phages from the transient stocks were propagated with the corresponding host M. TB isolates and the representative ATCC reference strains using the plate method as follows: Ten folds serial dilutions (10^{-1} to 10^{-6}) were made with Lambda buffer for the phage stock solutions by taking 100 µl of the phage solution into 900 µl of lambda buffer. Transfer of 100 µl of each dilution for each phage stock solution into 15 ml volume sterile plastic container contain 100 µl of 10^9 colony forming unit (CFU) $ml^{-1}$of 2-weeks blood broth culture of targeted bacteria and incubate at 37ºC. After 10 min incubation, the added 2.5 ml of top layer agar cooled to 45ºC and poured over blood agar plates. Plates were incubated 10d at 37ºC and plaque morphology, ***growth characteristics were recorded according to the following parameters:***

Diameter (mm) of the plaque, Shape of the plaque, Depth of the plaque, Margin cut, Clarity or turbidity of the plaque, Plaque visible time. By conducting a thorough examination

of the formed plaques, it was found that only very few out of tens or hundreds plaques per single plate show larger diameters and clearer lysis than the average. The slightly larger plaques proved to be an excellent indicator for the optimization of the phages lytic characteristics. Accordingly, the best 3-5 well-defined, clear, and largest plaques were selected at each run and phages from these best plaques were selected for other runs as mentioned above. This passage-based enhancement of phages lytic characteristics has been repeated for 2 runs in order to magnify the outcome of the biased selection of the large and clear plaques till obtaining the largest and theclearest 3-5 plaques, which reached to the ceiling of plaque-based optimization, reflecting the best yet possible enhancement of the lytic characteristics of the bred phages (7, 13, 14).

Biokinetic-based optimization: This advanced optional step of optimization was carried out on the phages recovered from the 3-5 optimized plaques that resulted from the plaque-based optimization technique and showed a need for further optimization. The aim of this biokinetic-based optimization is to investigate the biokinetics of the resulted 3-5 optimized phage sets. Moreover, this step was used to choose the phage set which shows the highest biokinetic values given that remarkable differences in the biokinetic values were seen among the tested phage sets which might be overlooked by the previous plaque-based optimization technique.

1) Design and standardization of the viricidal assay: The biokinetic assay is based on neutralizing/destroying extracellular phages after certain contact time (the time needed for phages to attach to bacterial cells and inject their nucleic acid) without harming host bacterial cells. After numerous experiments, once the contact time is elapsed, the extracellular phage particles were neutralized by harsh vortexing for 20 sec at room temperature. This step destroys the tail fibers of the phages still outside host bacterial cells rendering them unable to infect further cells while phage-infected host bacteria still intact as 20 seconds are not enough to destroy them.

2) Design of the biokinetic assay: Ten (10) µl, 10^{12} plague forming unit per ml (PFU ml^{-1}) of anti-mycobacterial phages and 10 µl of bacteria (10^5 CFU ml^{-1}) were mixed for contact times 10, 20, 30, 40, 50, and 60 min. Then, vortexing for 20 sec at room temperature was done to neutralize extracellular phages. Then 1ml of blood medium was added. Ten (10) µl were transferred in microcentrifuge tube containing 900 µl Lambda buffer, so 10-fold serial dilutions were prepared. From each dilution, 10 µl were spotted on the appropriate bacterial lawn at timely intervals (1-6) hours to recover the formed plaques before and after the burst of the new phage progenies. The plates were then incubated at 37ºC for 12 days.

Interpretation of the biokinetic assay: The interpretation of the results was classified into two eras; the pre-burst era and post-burst-era. At the pre-burst era, the number of the PFU or plaques is equal or less to the number of the bacteria used in the test for the given dilution. At this era, each plaque was formed by lysis of one bacterial cell releasing high number of phage progenies in situ leading to formation of a plaque. That means each bacterial cell sheltered certain number of replicating phages which will then form a plaque. The time after the burst time is considered as post-burst era. At this era, each plaque represents a new phage progeny was released in the master tube before spotting onto the lawn. Hence in this assay plaques represent two meanings according to the pre- or post- era of the assay (7, 13, 14).

Therefore the interpretation will be as the following:

Phage binding time (PBT): The time for the encounter between bacterial hosts and their specific phages that gives the highest number of phage particles at the pre-burst era or yields the highest infective ratio.

Infective ratio (IR): it is the ratio between the number of phage particles at the pre-burst era and the number of the bacterial hosts used in the assay. IR=No. of phage particles in the pre-burst era at a given dilution / No. of the bacterial hosts used in the assay at the same given dilution. The closer number of plaques in the pre-burst era to the bacterial titre used, the higher the IR.

Burst time (BT): it is the time measured before a sharp increase was observed in the number of the formed phage particles more than the number of the bacteria used for the given dilution. In other words, it is the time when the new phage progenies became responsible for the formation of plaques rather than their infected host cells.

Chemical phage design (chemical/physical re-adaptation of the phage-host specificity)

The aim of the chemical design techniques is to breed new phage progenies by chemical/physical re-adaptation of their host specificities to become artificially lytic to new host bacteria that previously used to be completely resistant to the parent phage particles. Several chemical substances were used in controlled physical conditions to supplement cultures of phage-resistant M. TB bacteria mixed with certain number of non-specific phages to physically/chemically readapt attacking phages to infect and lyse the used-to-be phage resistant bacteria. The mixture of chemical substances at certain physical conditions is called the designing solution. The designing solution is designed to modify the structure and exposed moieties of the bacterial host cell wall and to change the integrity of M. TB wax-loaded cell

wall in order to crack and unexpose hidden moieties and structures within the cell wall of M. TB. Once the tail fibres and the baseplate of the attacking phage attach quite firmly to the newly recognized moieties, the insertion of their nucleic acids will be triggered immediately to pass through the cell wall into the interior of the bacterial cell and start the lytic infection process. The hypothesis of the current methodology of the chemical design of phages is to create an artificially-designed microenvironment, in the design solution, for the attacking phages to unusually succeed in infecting a naturally resistant strain of bacteria and produce altered phage progenies that acquire the specificity of the new host. It is hypothesized that once phage nucleic acid gets inside phage-resistant bacteria, there will be a possibility of gaining genes from prophages integrated in host cells relying on homologous recombination of closely related insertion sequences of bacteriophages. These genes might confer new specificities required for resigned phage progenies to specifically infect and lyse this particular bacterium in the future.

A number of chemical non-genetic designing protocols were conducted at the same time. The design of these protocols is dependent mainly on the concept of modifying, changing, and partially tearing the cell wall of the host bacteria to become artificially susceptible to phage infection. However, only three protocols were found to be fruitful for the current endeavour and they were used in this study main work.

Tween-80-based protocol:Tween 80 (Merck, Germany) is considered as an active substance against proteins and lipids. Different concentrations of Tween ***80 were tested from 0.4 to 10% as follows:***

0.4 to 10% v/v of tween 80 were used in 1 ml of two-weeks M.TB culture in sheep blood media in 10 ml sterile tube; 200 ul of total 5 optimized anti-TB phages were added at concentrations (10^{10} PFU ml^{-1}) per phage and incubated at 37ºC. Every 2d, 100 µl of 10 strengths of blood medium were added followed by the addition of 10 µl of each of the used 5 phage stocks and a loopful 2-weeks M. TB culture of the same target bacteria. In addition, every week, 10 ul diluted Tween 80 were added regularly. This was continued for three weeks and phage lytic activity was tested every 3 days when thin bacterial lawns of the same target bacteria were prepared and 10 µl of the Tween 80-treated phages were added on bacterial lawns and then incubated at 37º C for visualizing resultant plaques.

Sublethal dose of Mycobacterial lysis buffer:

Mycobacterial lysis buffer, or MLB (86.6 ml H2O, 8 ml 5 M NaCL, 2 ml 2 M TrisHCl (pH 8), 3 ml 20% SDS, 400 µl 0,5 M EDTA, 220 µl 15.6 mg/ml proteinase K (Roche Diagnostics, Switzerland) was used in this method (15). Sublethal dose of MLB used with M. TB, MLB diluted in 1M TrisHCL 10% (v/v) was shown to be the optimal sublethal lysis buffer for mycobacterial cells where no more than one log reduction of living M. TB was seen.

About 300 ul of diluted MLB were mixed in 1 ml of two-weeks culture of M.TB in sheep blood broth in 10 ml sterile tube; 200 ul of total 5 optimized anti-TB phages were added at concentrations (10^{10} PFU ml^{-1}) per a phage and incubated at 37ºC. Every 2d, 100 µl of 10 strengths of sheep blood broth were added followed by the addition of 10 µl of each of the used 5 phage stocks and a loopful 2-weeks M.TB culture of the same target bacteria. In addition, 10 ul of diluted MLB were added regularly every week. This was repeated for three weeks. Phage lytic activity was tested every 3d when thin bacterial lawns of the same target bacteria were prepared and 10 µl of the MLB-treated phages were added on bacterial lawns and then incubated at 37º C for visualizing resultant plaques.

Xylene phage-based design technique: Xylene is known as an efficient lipid-solving and dewaxing agent. Since the cell wall of acid fast bacilli is loaded with wax and lipoproteins, the use of xylene is tested for modifying mycobacterial cell walls. However, xylene can kill the exposed bacterial cells at certain concentrations. According to the trial and error experiments done in this study, the lethal dose of xylene for M. TB was found to be 4% v/v. Hence, 0.8% v/v of xylene was found to be optimal as sublethtal dose which can be used for destabilizing the M. TB waxy cell wall. Accordingly, the phage design solution was made using constant elements including the sublethal dose of 0.8% xylene, 2-weeks culture of one of the TB isolates at $1x10^9$ CFU ml^{-1}, a mixture of 4-5 optimized anti-TB phages at 10^{10-12} PFU ml^{-1}, and 4 to 46 mM Tris-HCl buffer (pH 8). The phage design mixture was incubated for as long as required (90 days were tried) at 37ºC with subsequent addition of loopful 2-week fresh culture of M. TB and 100 µl of the desired phages (10^{12} PFU ml^{-1}) every 3d. In addition, 0.04% v/v was added every week for 3 weeks. Phage lytic activity was tested every 3d for visualizing any resultant plaques.

Results:

Phage isolation and plaque- and biokinetic- based optimization

A total of five phages were isolated. Three phages (Mtb-1P to Mtb-3P) were isolated and optimized to three isolates of M. TB (Mtb-1 to Mtb-3). In addition, two phages (25177-P and 25618-P) were isolated and optimized to two reference strains of M. TB, namely, ATCC 25177 and ATCC 25618 while no phage was found to ATCC 27294 (Table 1).

Table 1: The optimization of the isolated wild phages against M. TB isolates in terms of plaque and biokinetic methods

Isolate of M. TB	Crude specimen of the phage	Phage name	Before optimization				After optimization			
			Plaques		Biokinetic		Plaques		Biokinetic	
			Size (diameter; mm)	Clarity	IR (%)	BS	Size (diameter; mm)	Clarity	IR (%)	BS
Mtb-1	Soil	Mtb-1P	1.6	SC	83	79	2.8	CL	92 P<0.05*	136 P<0.05
Mtb-2	Sewage	Mtb-2P	1.2	CL	85.2	122	2.4	CL	91.3 P>0.05	152 P<0.05
Mtb-3	Sewage	Mtb-3P	2.1	SC	75.7	78	3.1	CL	93.4 P<0.05	138 P<0.05
ATCC 25177	Soil	25177-P	1.4	SC	86.4	94	2.3	CL	95 P<0.05	115 P>0.05
ATCC 25618	Sewage	25618-P	0.8	SC	88.1	116	2.4	CL	91.8 P>0.05	169 P<0.05

*: P value when compared with corresponding figure before optimization
-CL: clear plaque -SC: semi-clear plaque -ST: semi-turbid plaque
-TR: Turbid plaque -BS: Burst size -IR: infective ratio

Table 2: Chemically designed anti M.TB phages along with their plaque and biokinetic parameters

Anti M.TB designed phages	Plaques		Biokinetic	
	Size (diameter; mm)	Clarity	IR (%)	BS
Mtb-1RP	1.1	SC	80.5	96
Mtb-2RP	1.3	CL	89.1	117
Mtb-3RP	1.4	SC	90.2	78
Mtb-4RP	0.6	SC	74.6	108
Mtb-5RP	1.0	CL	81.4	110
Mtb-6RP	0.8	SC	86.9	105

Accordingly, a mixture of anti-M.TB phages was progressively built, the phage master mix. The parameters of plaque-based and biokinetic- based optimization of the anti-M.TB lytic phages are shown in Table 1. The results of the plaque- and biokinteics- based optimization for the isolated phages were highly promising in terms of the phage plaque criteria and in the phage biokinetic values. Since the biokinetic values, the burst time (BT) and the optimal phage binding time (PBT) showed no remarkable differences before and after optimization techniques, only the burst size (BS) and the infective ratio (IR) were shown in this table. Most of the phages isolated and optimized from the clinical isolates and reference strains of M. TB (Table 1) showed significantly enhanced lytic potential in terms of plaque size and clarity and in burst size and infective ratio rate ($P<0.05$). Moreover, the increments in the IR, BS, and plaque size values after the optimization were correlated positively with each other. The overall correlation coefficient between the increments of IR and BS was $r = +0.63$, and between the increments of BS and plaque size was $r = +0.72$, and between the increments of IR and plaque size was $r = +0.57$ ($P<0.05$). This provided further consistency of our optimization techniques that three parameters for the phages lytic cycle optimized similarly and correlated with each other significantly.

Phage biokinetics : Phage growth was characterized by the PBT, BT, BS, and IR (determined all in modified one-tube growth experiments). The results showed that all isolated phages from the optimization experiments (Table 1) have an optimal PBT to host cells of 20 min with average BT of 2h with non-significant difference in BT between the phages before and after optimization ($P>0.05$). On the other hand, mean BS after optimization was 142.4. BS showed great variance among the tested phages and showed a significant difference between pre- and post-optimzation phages. The minimal optimized BS was 78 phage particles per a cycle and the maximal burst size was 152 phage particles per cycle. One of the most important parameters of the phage biokinetics is the infective ratio (IR) in which it was found highly variable among the tested phages as well as significantly different between pre- and post- optimization phages. Therefore, the results of the current phase of the study showed that the characteristics of lytic cycle of the optimized anti- M.TB phages were enhanced significantly after plaque and biokinetic- based optimization for M. TB bacteria. Accordingly, the optimized phages turned out more lytic with higher burst size (enhanced phage replication), higher IR (enhanced infective capability), and higher clarity and size of resulted plaques. This is a very essential step for the success of phage biocontrol and/or therapy to M.TB. Moreover, it is important to use highly optimized phages for the coming steps of chemical design techniques and maybe future phage lysin extraction.

Chemical designing of optimized lytic anti M.TB phages: Three techniques succeeded in designing phage to host bacteria specificity and they were applied on 40 isolates and 3 reference strains of M. TB separately using the idolsted and optimized phages that are normally not lytic to a target bacteria. Each technique was performed by using combinations of scalable components in order to cover all

chances of the optimal phage designing protocols. Extended time periods, 90 days, of phage designing techniques were pursued. Tween-80, MLB, and xyelene techniques succeeded in designing 1/40, 4/40, and 1/40 of phage:host bacterial pairs, subsequently. MLB technique was shown to be the most effective method for reorienting phage: bacteria specificities (Table 2). Hence, six new phages (4 wild phages and two reference bacteria phages: 25177-P and 25618-P) whose specificities were reoriented to new host bacteria, were formed. It is noteworthy to mention that the newly designed phages lost their specificities to the original host bacteria. The new six phages were named as Mtb1RP-, Mtb6-RP. The plaque and biokinetic characteristics of the new designed phages are shown in (Table 2).

Discussion:

The fight against tuberculosis has not settled with fears that the battle will be for the side of the disease. Emergence of new strains of MDR, extensive drug resistant (XDR), and pan drug resistant (PDR) M.TB rendered the mission of scientists and health bodies all over the world more difficult (16). It is not a secret that the rate of producing new anti-TB drugs is slower than the rate of emergence of new MDR, XDR, and PDR M.TB (17). Therefore, it is prudent nowadays to search for alternative sterilizing, biocontrol and therapeutic strategies to combat tuberculosis in case the worse comes to worst. According to this sense, this study was conducted in endeavor for finding new approaches of using lytic phages for the treatment of tuberculosis instead of or as adjuvant with anti-TB drugs. In addition, highly lytic mycophages can be the ultimate source for producing anti-TB phage endolysins that act as novel anti-TB medicines. Although M.TB is an intracellular pathogen, using mycophages in vivo was shown to be decisive for curbing disease spread especially in miliary TB and locally wide-spread active forms of disease (18). Moreover, specific anti-TB phages can be used as diagnostic tools for TB bacilli as well as screening TB resistance to anti-TB drugs (6, 19-21). One of the drawbacks of using phages for biocontrol, therapy, or diagnosis of TB is the difficulty to find highly lytic and virulent phages to M.TB (18). It is well known in the field that M.TB phages are less virulent and lytic than Gram positive or negative bacteria (18). Therefore, optimization of the isolated phages to M.TB seems interesting. For this reason, we used plaque-based passaging and biokinetic-based passaging methods for optimizing wild anti-TB phages. This method was tested before on Gram negative and positive bacteria (7, 13). However, it was not tested on M.TB. This study showed that plaque-based and biokinetic based optimization techniques were fruitful in enhancing significantly plaques' size and clarity as well as increasing phage burst size and infective ratio (Table 1). The enhanced parameters indicate enhanced virulence of these phages making their use in anti-TB more promising. The other drawback of using anti-TB phage therapy is the relatively rapid emergence of resistant bacteria to attacking phages (2, 8). Therefore, it was smart to think in using phage cocktails or phage lysins in order to override this setback. However, finding highly lytic and specific phages to form anti M.TB phage cocktails is not an easy and rapid task due to the long generation times of this bacteria and because of it is unique cell wall (21). So, the need to find new approaches for getting the proper number of lytic anti-TB phages seems vital to win this battle against the disease. One of the hypothesized approaches in this study is to accelerate finding new phages for problematic strains of M.TB by finding out a method to reorient phage specificity to target M.TB. The hypothesis of this study was based on a notion that if it is possible to actively in-lab, introduce an anti-TB phage to a certain strain of M.TB cell that is not susceptible to that phage, the phage once introduced will grow inside the bacterial cell and the phage progenies might get altered specificity enabling them to infect that bacteria passively. The challenge was to find the proper methodology to introduce anti-TB phages. It was figured out that this is possible if we insult and modify M.TB cell wall without killing them. From this conception, the idea of conducting chemical designing of phages came through. Three methods were proven to be successful out of 8 methods tested. The current study showed that MLB was superior in designing anti-TB phages over the other two methods, namely, Tween-80 and xyelen. It is not clear why MLB did better than others but it might be attributed to its superior modifying potential on M.TB cell wall. This study is the first to achieve successful chemical anti-TB designing since previous studies were done only on Gram negative and positive bacteria (13, 14). The patenting of new techniques of anti-M.TB phage designing is under process.

Conclusions:

Taken together, it is concluded that optimization of phages along with successful phage designing might open doors wide to the field of phage-based therapy, biocontrol, bioprocessing, and rapid diagnosis for bacteria in general and for MDR or XDR M.TB in particular. It is suggested that combination of phage optimization plus designing would render using anti-TB phage cocktails feasible. Phage cocktails to M.TB are believed to be one of the ultimate goals for successful phage therapies, biocontrol or rapid diagnosis of TB.

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