The Antimicrobial and Synergistic Efficiency of Antibiotic and Bacteriophage for the Treatment of Tuberculosis

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Bio:

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Abstract:

Mycobacterium smegmatis mc^{2}155 is a commonly used model organism for *Mycobacterium tuberculosis*, which is one of the most serious bacterial infections in the world. This study explored bacteriophages as potential treatment, and it was proposed that due to each agents' distinct mechanisms of action, the synergy of antibiotic (isoniazid and ethambutol, separately), and bacteriophage would provide for more effective treatment than either agent alone in treating *Mycobacterium smegmatis* over time. Increasing concentrations of antibiotic were diluted with varying concentrations of lytic mycobacteriophage D29 and compared to the activity of the same varying concentrations of antibiotic and bacteriophage D29 alone on *Mycobacterium smegmatis* over time using absorbency values. While both agents only significantly treated the bacterial concentrations, bacteriophage in combination with ethambutol was significantly more effective than either agent alone, suggesting that the use of bacteriophage in combination with specific antibiotics should be explored further, especially for treating multi-drug resistant tuberculosis.

Introduction

Mycobacterium tuberculosis, the primary causative agent of tuberculosis, was once thought to be nearly eradicated. However, due to a multitude of causes such as the rise of the AIDS epidemic and poor administration of antibiotics, today it has reestablished itself to be the second leading cause of death globally and currently causes 1.5 million deaths annually [1]. *M. tuberculosis*, however, has become increasingly difficult to treat due to its high levels of antibiotic resistance to many first-line and second-line drugs. It is essential that viable treatment for *M. tuberculosis* remain, as the disease can cause various complications such as lung failure, relapse of the disease, impaired kidney and heart function, joint destruction, and can lead to significantly increased risk of acquiring meningitis, cardiac tamponade, and immunodeficiency diseases such as HIV/AIDS [2].

Currently, the primary treatment for *M. tuberculosis* is first-line antibiotics, including isoniazid, rifampicin, pyramidizine, and ethambutol. However, *M. tuberculosis* has and continues to evolve antibiotic resistance against these drugs, and it remains a problematic therapeutic target due to its capacity for dormancy and cell wall composed of mycolic acids, which can be difficult to penetrate [3]. Recently, many cases of multi-drug-resistant tuberculosis have been reported. Consequently, a different approach towards treating *M. tuberculosis* is highly desirable.

Bacteriophages are viruses that specialize in lysing, or bursting, both antibiotic resistant and antibiotic-sensitive bacteria and have been used successfully to treat bacterial infections in Eastern Europe for almost a century. Therefore, they are a potential alternative treatment for bacterial infections in the United States as well. Bacteriophage has been shown to be efficient independently in treating *Mycobacterium smegmatis*, a commonly used model organism. The

cell wall compositions are also similar between the two bacteria as they are both composed of mycolic acids.

Mycobacteriophage D29, specifically, has been demonstrated to show significant efficiency in treating *M. tuberculosis* [4]. Mycobacteriophage D29 also has significant potential to treat other mycobacteria diseases, such as *M. ulcerans* and *M. avium* and has been demonstrated to effectively lyse these bacteria *in vivo* [5].

However, significant research has not been conducted into the use of bacteriophage and antibiotic in synergy. As bacteriophage and antibiotics have distinct mechanisms of action for treating bacteria, the use of bacteriophage and antibiotic together should be compared in efficiency to that of bacteriophage and antibiotic independently. It was expected that bacteriophage and antibiotic in synergy with higher concentrations of bacteriophage and lower antibiotic, comparatively, would be the most efficient in comparison bacteriophage and antibiotic alone for treating *Mycobacterium smegmatis* as a model for tuberculosis.

Materials and Methods

Bacterial strains and growth conditions

Mycobacterium smegmatis mc^2155 was provided by the Hughes Microbiology Laboratory and purified prior to use through repeated streaking and colony isolation. *M. smegmatis* was grown in 7H9 broth supplemented with calcium chloride and deionized water and incubated for 72 hours in a shaker incubator at 250 rpm prior to subculturing. CFUs of $1x10^8$/mL were attained using a UV-mini 1240 spectrophotometer. Optical density values of 0.08-0.1À were accepted. *M. smegmatis* colonies were cultured using Difco Luria agar base a common agar base for the culturing of mycobacteria. For experimentation, bacteria were incubated at 37°C in 7H9 broth in the shaker incubator at 250 rpm. Samples were taken every 4.5 hours for 24 hours. Cell counts were measured using OD values.

Bacteriophage

Preparation. Mycobacteriophage D29 was also provided by the Hughes Microbiology Laboratory. Freeze-dried bacteriophage immersed in glycerol was reactivated by streaking the 1 mL of bacteriophage across the Difco Luria agar base and pipetting a solution containing 4.5 mL top agar and 0.50 mL *M. smegmatis* onto the base agar through the soft agar layer method. After incubation, plaques were determined to be pure and were inoculated into solution.

Creating a high-titer lysate. A plaque assay was conducted to calculate titer. Using the titer value, a web assay was conducted for the plaques to form a web over the plate. Web values were 0.25x, 0.50x, 1x, 2x, 5x, and 10x the PFU necessary to cover the plate. The web was then flooded with 5 mL of bacteriophage buffer and purified to attain the high-titer lysate (Figure 1). A titer above $1x10^7$ PFU/mL was considered to be a high-titer lysate.

Antibiotic

Determination of MICs. The MICs were calculated by the broth macrodilution method [6]. Because typical isoniazid MIC values range from 0.02 to 0.06 µg/mL, isoniazid was diluted to 0.2, 0.1, 0.05, 0.0025, 0.00125, 0.005625, and 0.00023125 µg/mL in 7H9 broth for MIC testing [7]. The concentrations were then doubled in order to maintain the concentrations after the addition of 1 mL of *Mycobacterium smegmatis*. The inoculum was from a primary 7H9 sample calculated to have a 0.5 McFarland. The solutions were then incubated in the shaker incubator at 250 rpm. Growth was assessed at 72 hours and defined as the first tube without clear growth. Because MIC values for ethambutol typically hover from 0.06 to 0.125 for mycobacteria, the broth-dilution method was repeated with ethambutol, with two-fold dilutions from 16 to 0.0078125 µg/mL [8]. The MIC value was used to calculate 0.25x, 0.50x, and 0.75x the MIC to be inoculated with *M. smegmatis*. This was conducted for both isoniazid and ethambutol.

Preparation of bacteriophage and antibiotic

Mycobacterium smegmatis was diluted with the above concentrations of bacteriophage and isoniazid. Bacteriophage and isoniazid were concentrated to derive the desired concentrations after the addition of 9 mL of *M. smegmatis* at $1x10^8$ CFU/mL. All three concentrations of bacteriophage were combined with the three concentrations of isoniazid. Bacteriophage and isoniazid were also used independently at the three different concentrations. The same was done for ethambutol. Nine mL of *Mycobacterium smegmatis* at a CFU of 10^8/mL in addition to 1 mL of 7H9 broth was used as the positive control. Ten mL of 7H9 broth were used as negative control.

Results

Agents only

Treatment with antibiotic alone. Concentrations of 0.25x, 0.50x, and 0.75x the MIC (0.0625 and 0.025 µg g/mL as MICs, respectively) were used for ethambutol and isoniazid. Ethambutol and isoniazid alone both demonstrated significantly decreased bacterial concentrations in comparison to the positive control, showing that the antibiotics are more effective in treating bacteria than no treatment (Figure 6 and 10, respectively). Isoniazid alone significantly decreased bacterial concentrations from initial concentrations (Figure 19). Ethambutol alone did not significantly decrease bacterial concentrations from the initial concentration (Figure 14). Antibiotics at higher concentrations were generally more effective than lower concentrations

Treatment with mycobacteriophage D29 alone. Bacteriophage D29 alone significantly decreased the initial concentration of bacteria, and by 22.5 hours, bacteriophage D29 had completely treated all of the bacteria, with average OD values of 0 (Figure 18). All PFUs (10^4, 10^2, and 10^1 PFU per mL) of bacteriophage demonstrated similar efficiency in treating bacterial concentrations (Figure 11).

Synergy of Antibiotic and Mycobacteriophage D29

Synergy of Isoniazid and Mycobacteriophage D29. Isoniazid and bacteriophage D29 in combination significantly decreased the initial concentration of bacteria for synergies of 0.50x and 0.75x MIC in combination with PFUs of 10^2 and 10^4 per mL and all bacteriophage PFUs, respectively (Figures 7-9). All isoniazid and bacteriophage D29 synergies were significantly more efficient than the positive control (no treatment) (Figures 15-17). It was observed that higher PFUs in synergy with higher isoniazid concentrations were the more effective synergies.

Synergy of Ethambutol and Mycobacteriophage D29. Ethambutol and bacteriophage D29 significantly decreased the initial concentration of bacteria at all ethambutol and

bacteriophage concentrations (Figures 3-5). Ethambutol and bacteriophage D29 demonstrated extremely significant efficiency in treating all the bacteria, having completely treated the bacteria by 18 hours (Figures 11-13). The synergy of ethambutol and bacteriophage was most efficient at lower concentrations of ethambutol and higher concentrations of bacteriophage (Figure 11), although all were significantly more efficient than all other treatments.

Conclusion and Future Work

Antibiotic alone, bacteriophage alone, and the synergy of antibiotic and bacteriophage were assessed for efficiency. It was found that all three different treatment types significantly decreased bacterial growth. However, bacteriophage in combination with ethambutol inhibited and decreased bacterial growth with highest efficiency, having completely treated bacteria by 18 hours. While isoniazid alone, bacteriophage alone, and isoniazid at 0.25x, 0.50x, and 0.75x the MIC in combination with all PFUs of bacteriophage significantly decreased the concentration of bacteria, ethambutol in combination with bacteriophage completely treated all bacteria, indicating that the synergy of antibiotic and bacteriophage should be considered for further therapeutic applications. All synergies, however, demonstrated significantly more efficiency than the antibiotic alone. All bacteriophage PFUs demonstrated no significant difference in efficiency (were equally effective), suggesting bacteriophage replication may accommodate for concentration. The OD (optical density) values and correlations were also supported by the initial and final plates.

These results concerning the efficiency of synergy support the findings in literature. For example, gentamicin and bacteriophage ATCC 19685-B1 (or Phage K) in synergy were more efficient than either agent alone in treating *Staphylococcus aureus* [9]. However, the distinct mechanisms of each agent are likely to affect efficiency of synergies. Ethambutol and isoniazid in combination with bacteriophage showed different efficiencies due to the distinct mechanisms of action for each antibiotic. This research shows that specific combinations must be sufficiently tested to determine whether the synergy would be more or less efficient than either agent alone.

Overall, however, the efficiency demonstrated by bacteriophage alone for treating *Mycobacterium smegmatis* and higher efficiency than isoniazid strongly suggests bacteriophage

may be used independently as well to treat both antibiotic-resistant and antibiotic-sensitive bacteria, especially *Mycobacterium* species and particularly, *M. tuberculosis*. Its significantly higher time efficiency suggests it has high potential for treating *M. tuberculosis*, as current drug regimens usually take six months to one year.

Continued research focusing on the delivery of bacteriophage to the host itself is important. Currently, bacteriophages are delivered via injection into the bloodstream. However, this decreases the efficiency of the bacteriophage due to the bacteriophage having to travel to the host. *Mycobacterium smegmatis* has the potential to be used as a carrier for a virulent phage. Once engulfed by macrophages containing the tuberculosis bacterium, the bacteriophage is directly deposited to its host. This has demonstrated over 95% more efficiency that bacteriophage alone [5]. Further research could develop this as a model and inactivate the *M. smegmatis* so that direct injection of the *M. smegmatis* does not pose a health concern. The delivery of bacteriophages could also be developed through the integration of bacteriophage into hand sanitizers, soaps, or daily antimicrobials. Research has shown that the addition of bacteriophage K into hand soaps decreased the concentration of *S. aureus* on hands by over 90% [10]. Bacteriophages have immense potential in medicine and health and should be further explored as antimicrobial agents.

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References

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Tables

Ethambutol Absorbency Values Over Intervals of Time for Varying Treatments

	Average Absorbancy Values Over Time (Hours)					
	0	4.5	9	13.5	18	22.5
Treatment Type						
0.75x MIC with 10^4 PFU	0.097	0.051	0.053	0.0265	0	0
0.75x MIC with 10^2 PFU	0.091	0.062	0.062	0.031	0	0
0.75x MIC with 10^1 PFU	0.095	0.050	0.060	0.030	0	0
0.5x MIC with 10^4 PFU	0.081	0.046	0.053	0.017	0	0
0.5x MIC with 10^2 PFU	0.090	0.046	0.067	0.034	0	0
0.5x MIC with 10^1 PFU	0.065	0.0503	0.054	0.025	0	0
0.25x MIC with 10^4 PFU	0.082	0.0408	0.016	0.008	0	0
0.25x MIC with 10^2 PFU	0.083	0.054	0.039	0.020	0	0
0.25x MIC with 10^1 PFU	0.091	0.043	0.043	0.022	0	0
10^4 PFU only	0.133	0.087	0.04	0.021	0.002	0
10^2 PFU only	0.133	0.095	0.036	0.0235	0.011	0
10^1 PFU only	0.125	0.1	0.043	0.025	0.006	0.006
0.25x MIC only	0.105	0.0803	0.060	0.086	0.011	0.125
0.5x MIC only	0.094	0.098	0.086	0.112	0.145	0.167
0.75x MIC only	0.103	0.078	0.065	0.086	0.107	0.123
Positive	0.107	0.144	0.142	0.169	0.196	0.197
Negative	0	0	0	0	0	0

*note that the time interval 13.5 hours is extrapolated

Table 1: Table of absorbance values over intervals of 4.5 hours for 24 hours for isoniazid and isoniazid combinations.

Isoniazid Absorbency Values Over Intervals of Time for Varying Treatments

	Average Absorbancy Values Over Time (Hours)					
	0	4.5	9	13.5	18	2.5
Treatment Type						
0.75x MIC with 10^4 PFU	0.093	0.0756	0.082	0.076	0.069	0.052
0.75x MIC with 10^2 PFU	0.099	0.076	0.083	0.077	0.07	0.07
0.75x MIC with 10^1 PFU	0.106	0.083	0.089	0.082	0.075	0.065
0.5x MIC with 10^4 PFU	0.19	0.074	0.072	0.065	0.059	0.052
0.5x MIC with 10^2 PFU	0.203	0.107	0.081	0.082	0.083	0.082
0.5x MIC with 10^1 PFU	0.1006	0.127	0.177	0.171	0.164	0.147
0.25x MIC with 10^4 PFU	0.104	0.088	0.153	0.159	0.164	0.132
0.25x MIC with 10^2 PFU	0.101	0.117	0.167	0.175	0.182	0.172
0.25x MIC with 10^1 PFU	0.094	0.147	0.203	0.185	0.166	0.188
10^4 PFU only	0.133	0.087	0.04	0.021	0.002	0
10^2 PFU only	0.133	0.095	0.036	0.023	0.011	0
10^1 PFU only	0.125	0.103	0.043	0.025	0.006	0.006
0.25x MIC only	0.122	0.122	0.070	0.085	0.099	0.099
0.5x MIC only	0.102	0.079	0.089	0.081	0.072	0.063
0.75x MIC only	0.096	0.079	0.13	0.097	0.063	0.050
Positive	0.126	0.093	0.230	0.230	0.234	0.29
Negative	0	0	0	0	0	0

Table 2: Table of absorbance values over intervals of 4.5 hours for 24 hours for ethambutol and ethambutol combinations.

Figures

Images

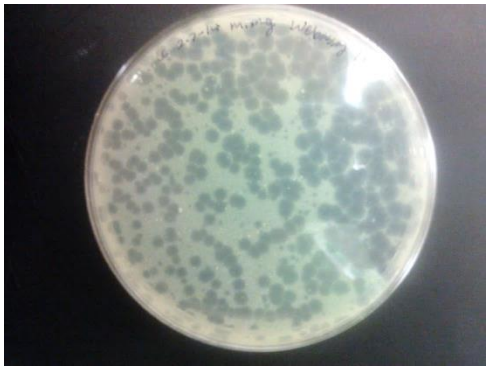

Figure 1: Web assay for attaining a high-titer lysate.

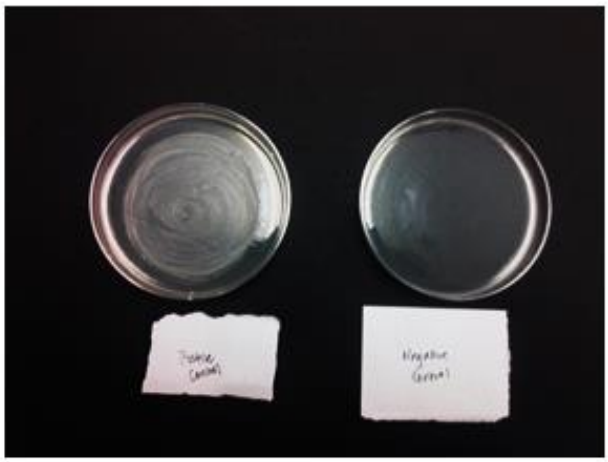

Figure 2: Positive and negative control.

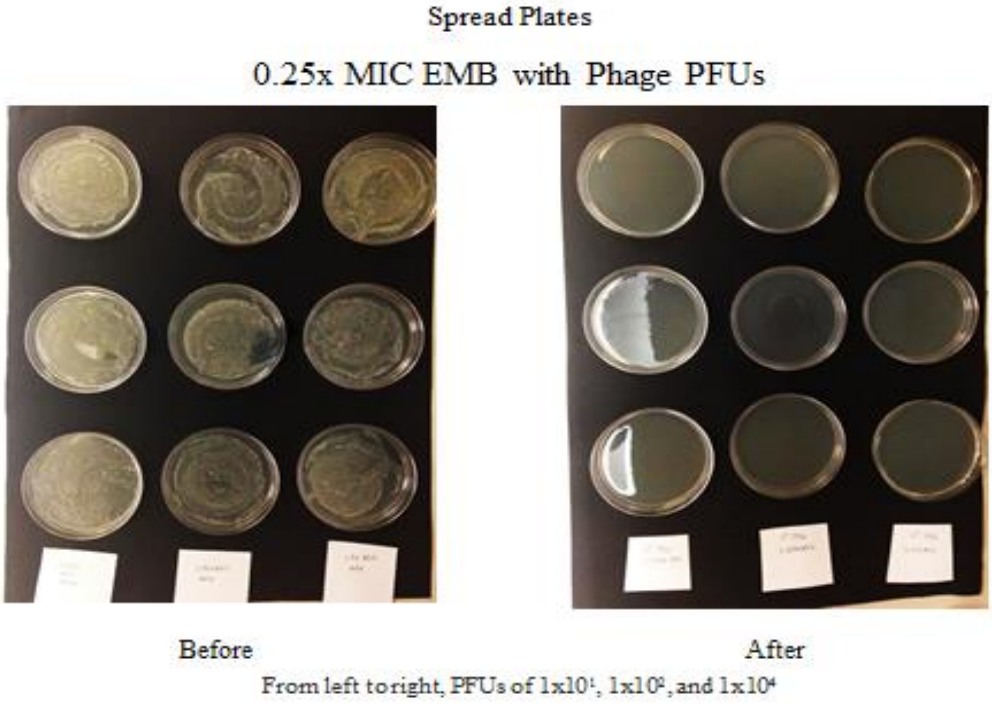

Figure 3: 0.25x MIC EMB and bacteriophage concentrations of 10^1, 10^2, and 10^4 PFU/mL. Spread plates of bacterial concentrations, before and after.

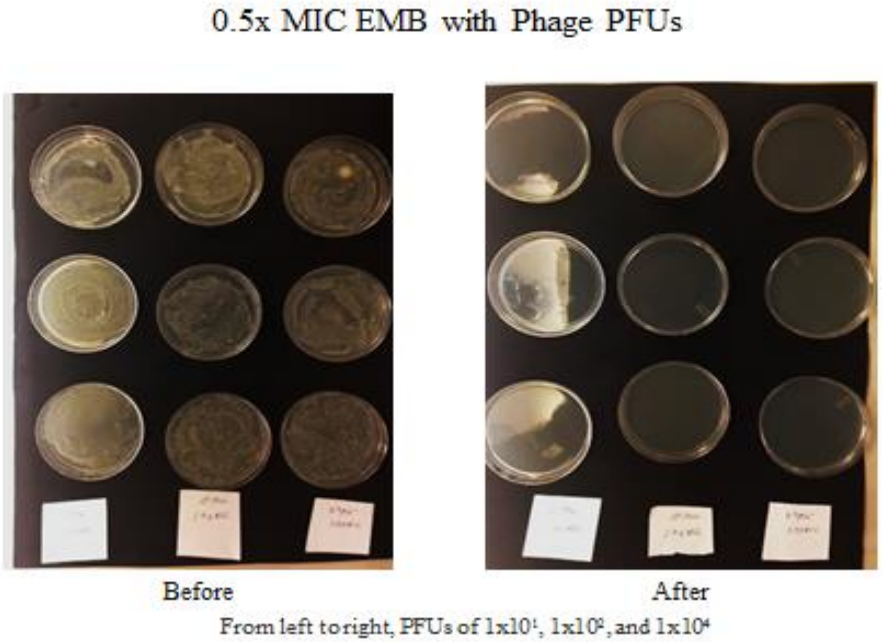

Figure 4: 0.50x MIC EMB and bacteriophage concentrations of 10^1, 10^2, and 10^4 PFU/mL. Spread plates of bacterial concentrations, before and after.

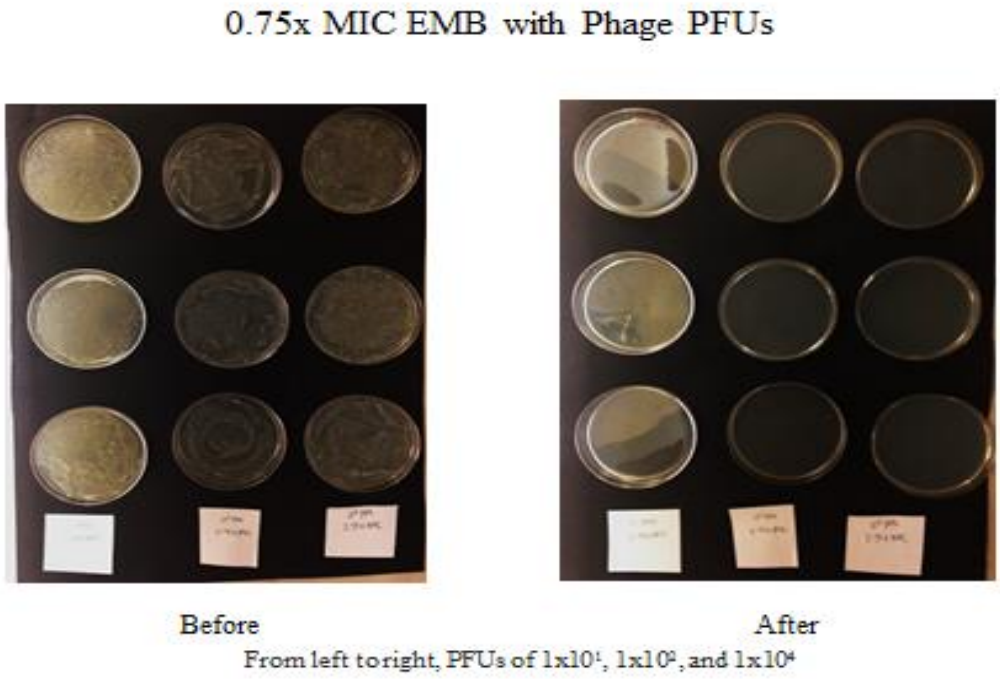

Figure 5: 0.75x MIC EMB and bacteriophage concentrations of 10^1, 10^2, and 10^4 PFU/mL. Spread plates of bacterial concentrations, before and after.

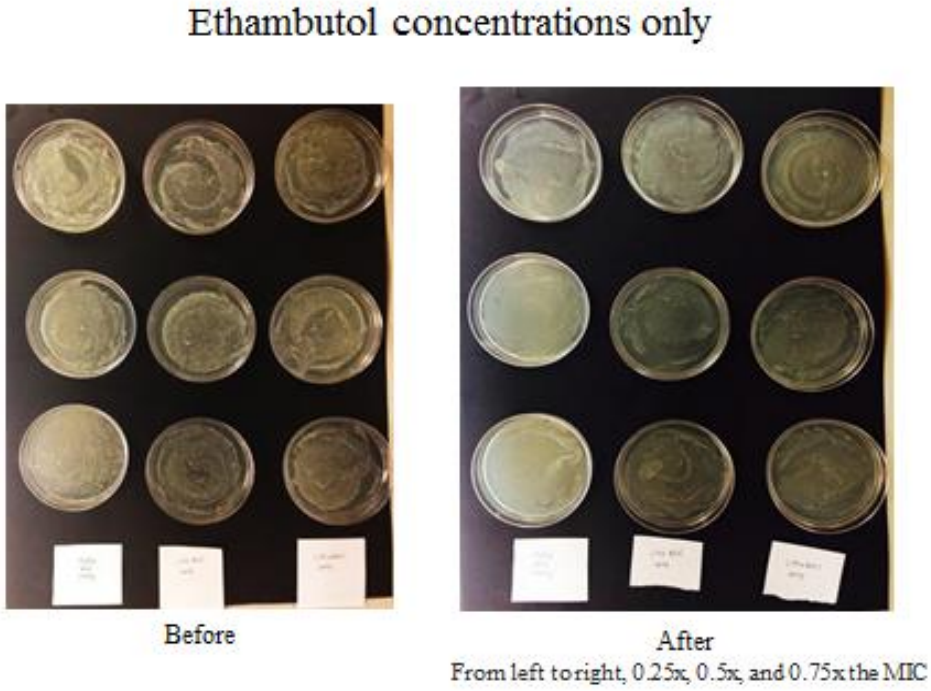

Figure 6: EMB only at 0.25x, 0.50x, and 0.75x the MIC. Spread plates of bacterial concentrations, before and after.

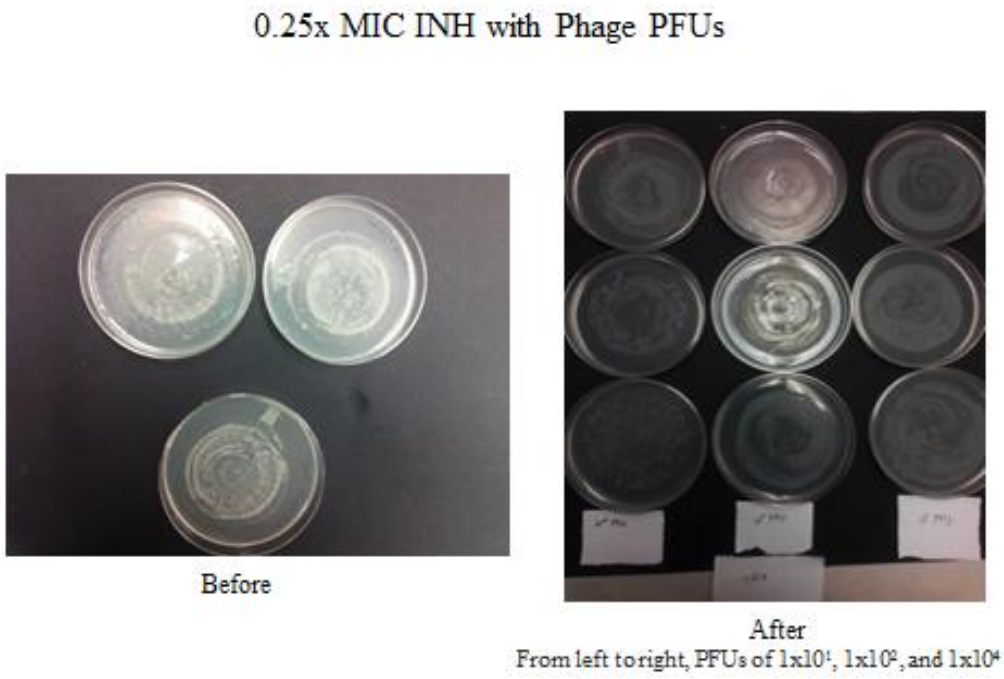

Figure 7: 0.25x INH MIC and bacteriophage concentrations of 10^1, 10^2, and 10^4 PFU/mL. Spread plates of bacterial concentrations, before and after.

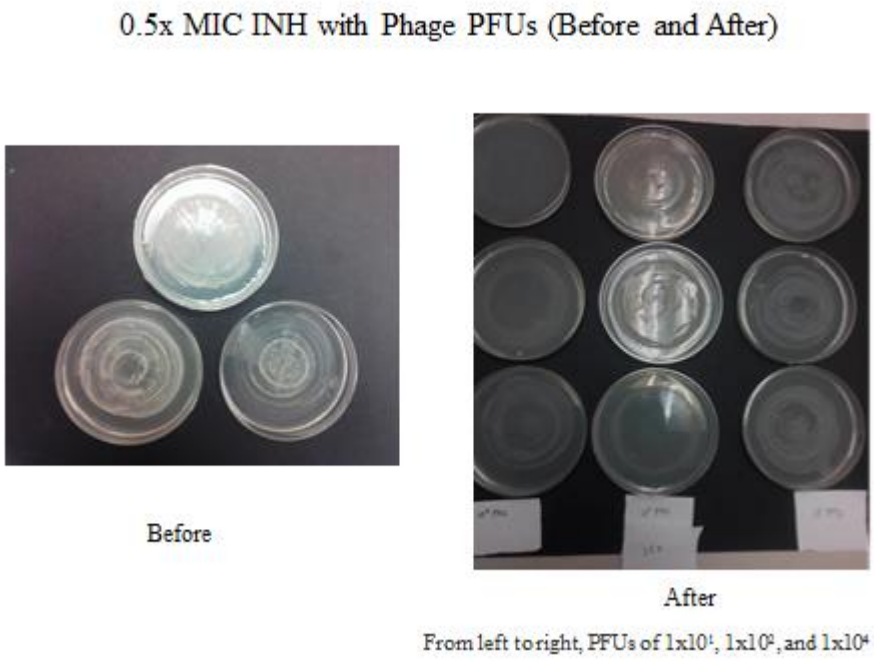

Figure 8: 0.50x INH MIC and bacteriophage concentrations of 10^1, 10^2, and 10^4 PFU/mL. Spread plates of bacterial concentrations, before and after.

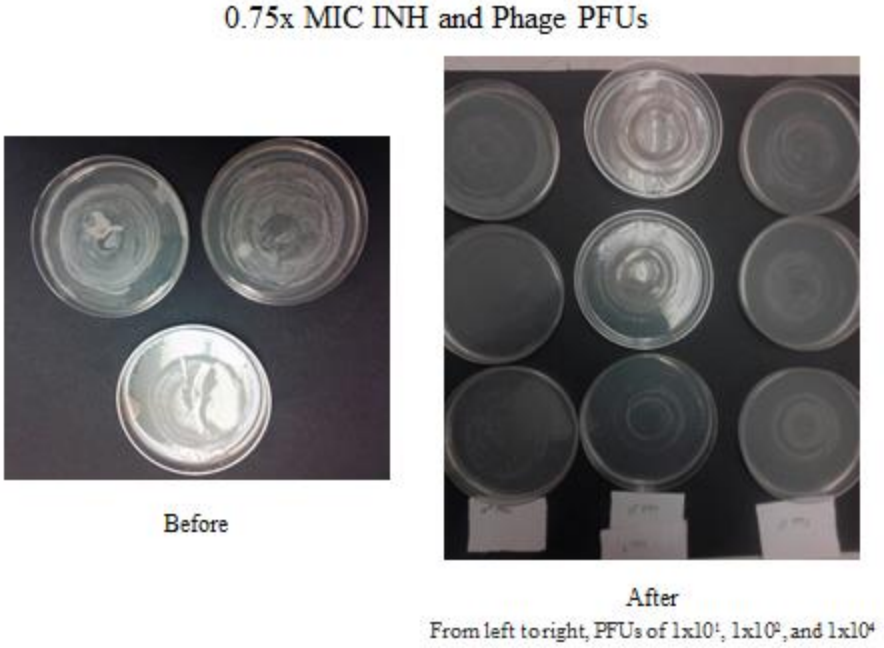

Figure 9: 0.75x MIC INH and bacteriophage concentrations of 10^1, 10^2, and 10^4 PFU/mL. Spread plates of bacterial concentrations, before and after.

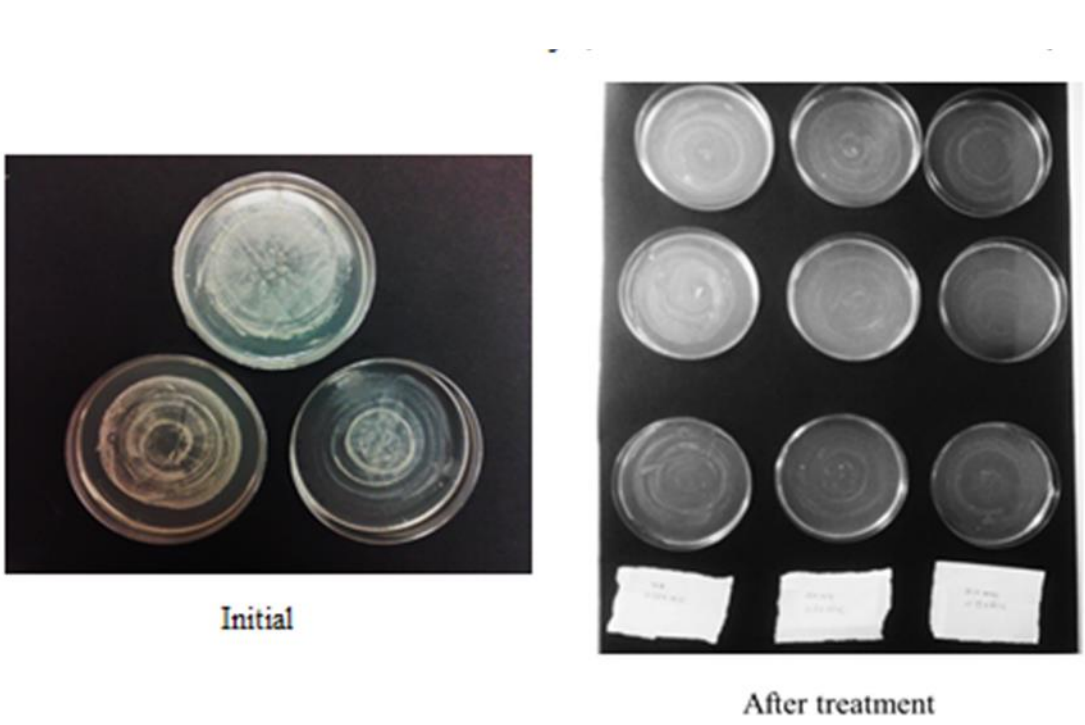

Figure 10: Isoniazid concentrations only at 0.25x, 0.50x, and 0.75x the MIC. Spread plates of bacterial concentrations, before and after.

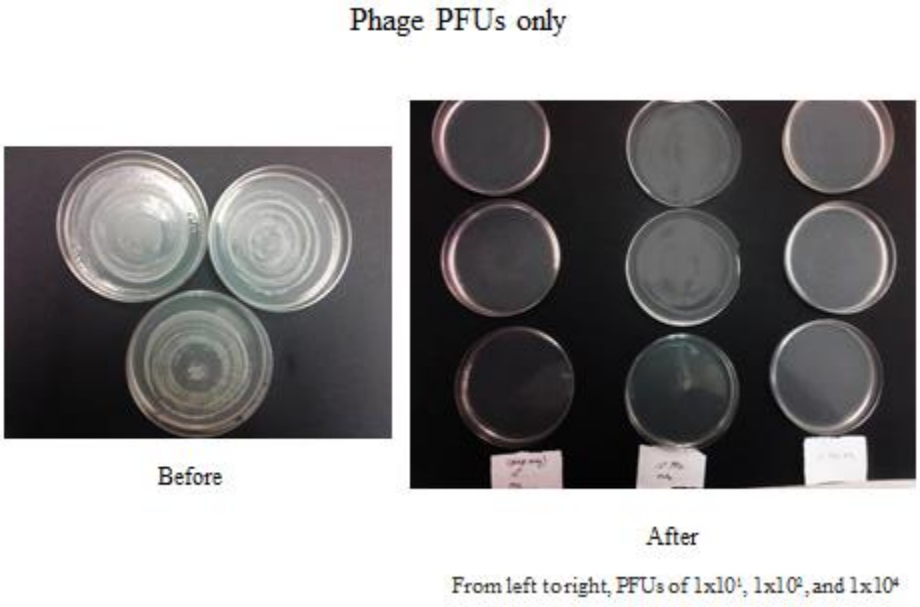

Figure 11: Bacteriophage PFUs only at PFUs of 10^1, 10^2, and 10^4/mL. Spread plates of bacterial concentrations, before and after.

Graphs

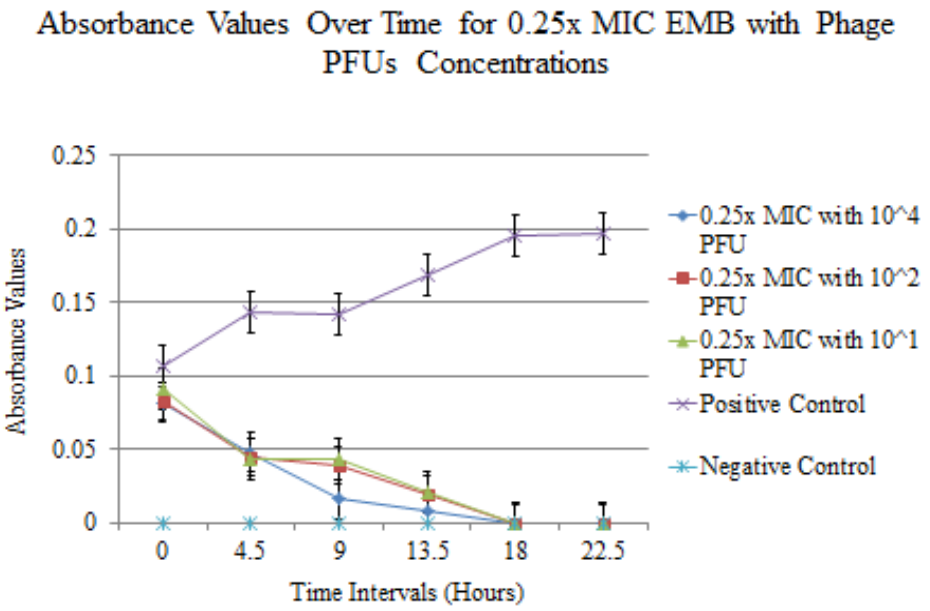

Figure 12: Absorbance values over 4.5 hour intervals for 0.25x MIC EMB in combination with PFUs of $1x10^1$, $1x10^2$, $1x10^4$ per mL.

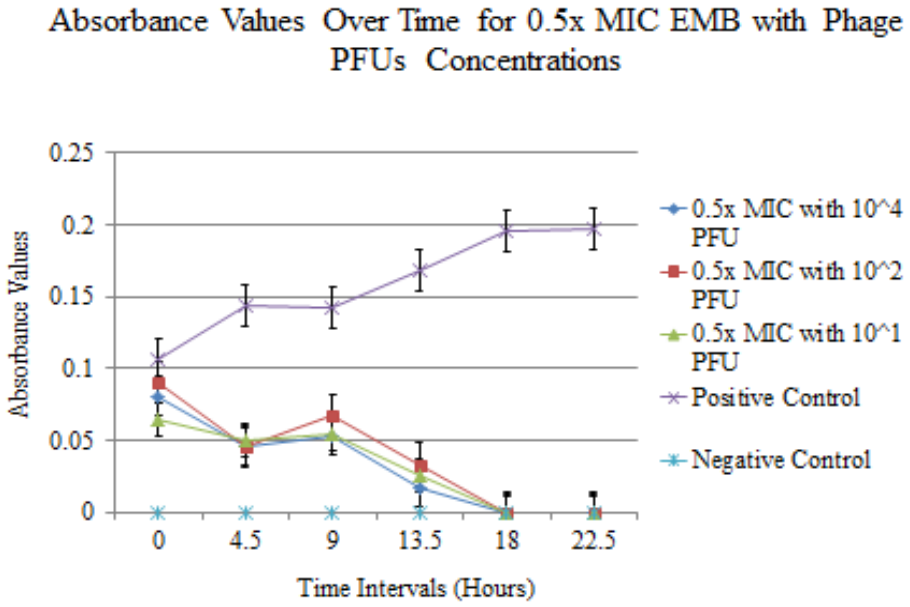

Figure 13: Absorbance values over 4.5 hour intervals for 0.50x MIC EMB in combination with PFUs of $1x10^1$, $1x10^2$, $1x10^4$ per mL.

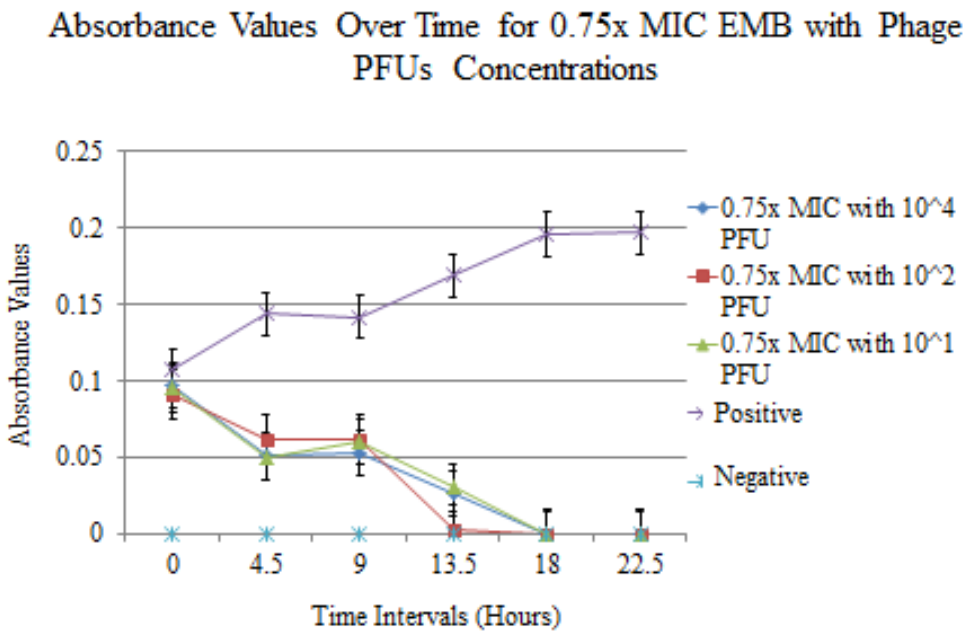

Figure 13: Absorbance values over 4.5 hour intervals for 0.75x MIC EMB in combination with PFUs of $1x10^1$, $1x10^2$, $1x10^4$ per mL.

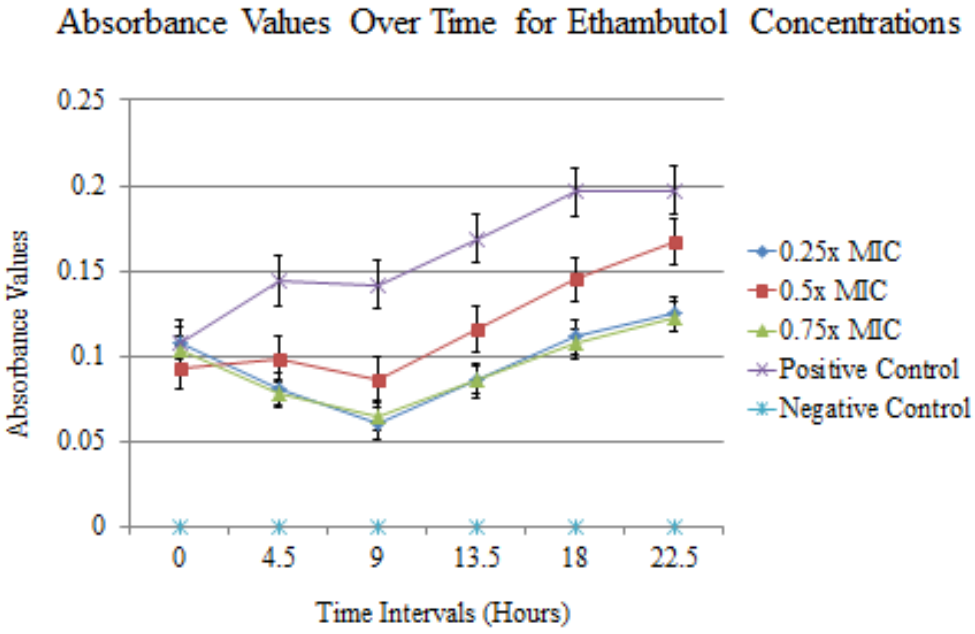

Figure 15: Absorbance values over 4.5 hour intervals for ethambutol only at 0.25x, 0.50x, and 0.75x the MIC.

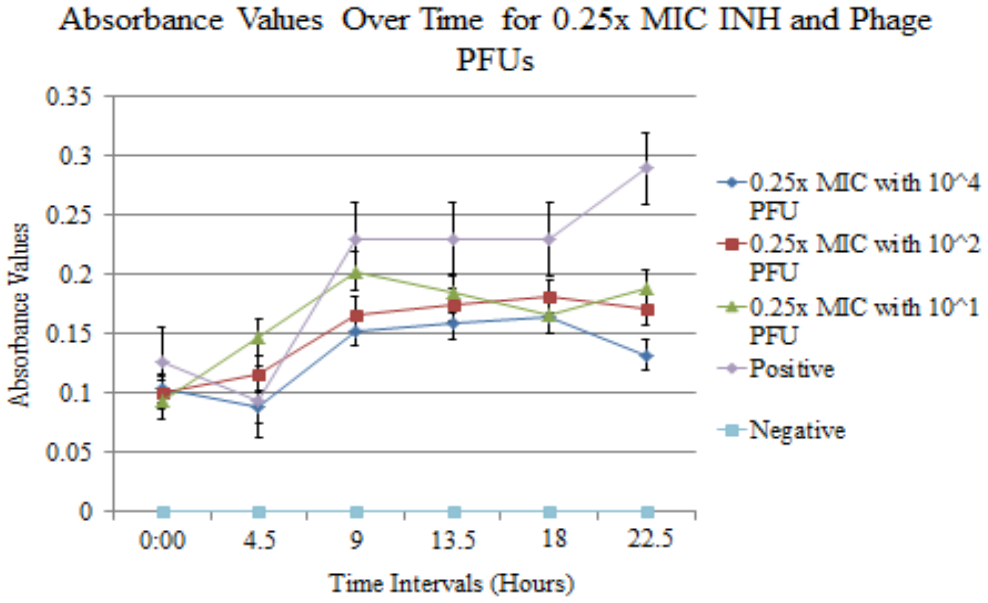

Figure 16: Absorbance values over 4.5 hour intervals for 0.25x MIC INH in combination with PFUs of $1x10^1$, $1x10^2$, $1x10^4$ per mL.

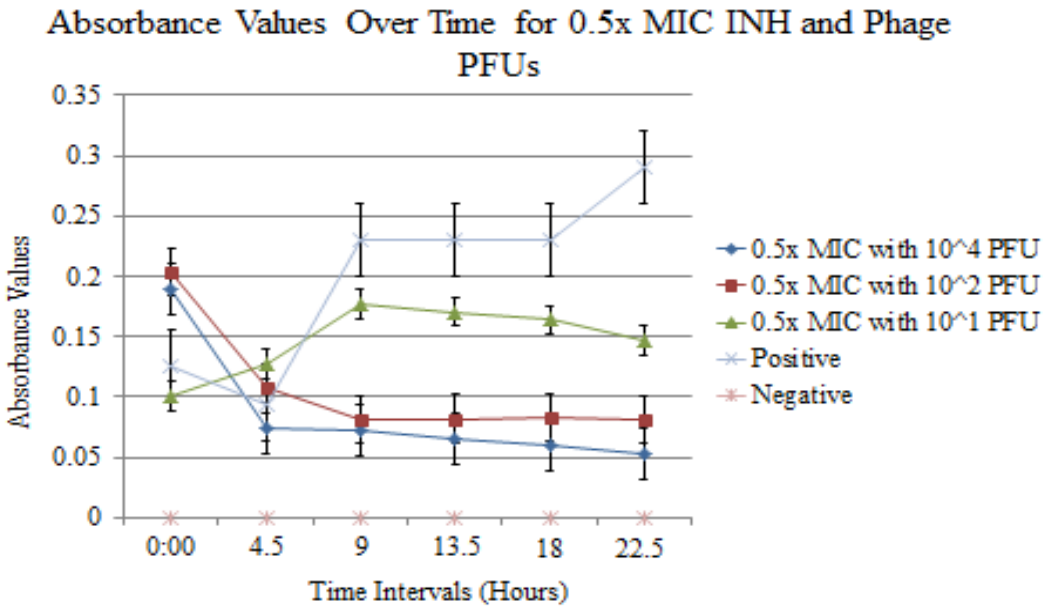

Figure 17: Absorbance values over 4.5 hour intervals for 0.25x MIC INH in combination with PFUs of $1x10^1$, $1x10^2$, $1x10^4$ per mL.

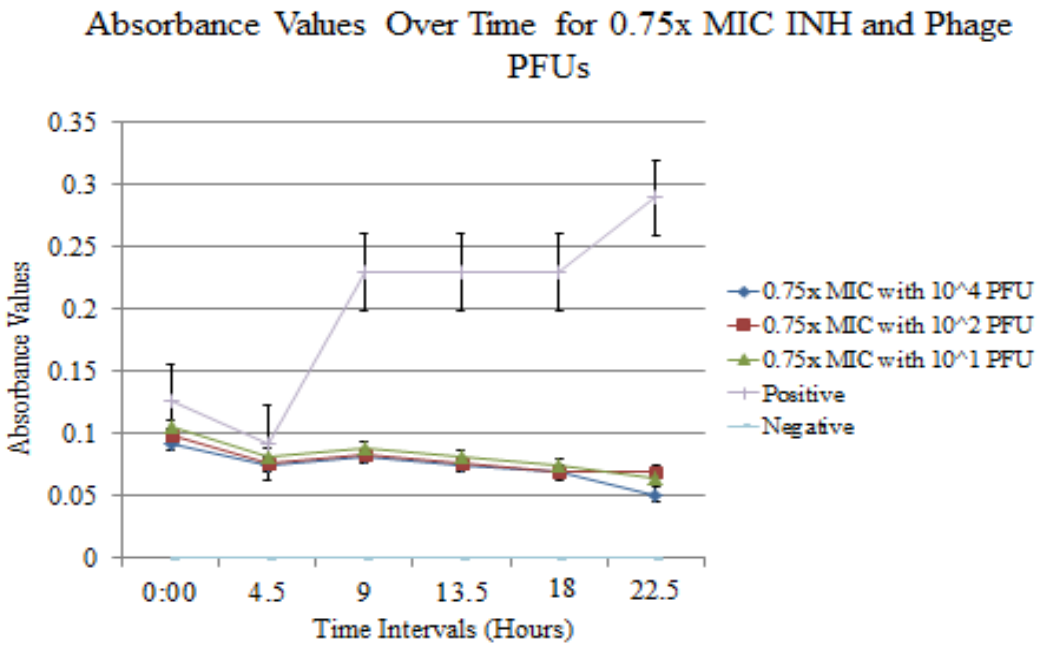

Figure 18: Absorbance values over 4.5 hour intervals for 0.75x MIC in combination with PFUs of $1x10^1$, $1x10^2$, $1x10^4$ per mL.

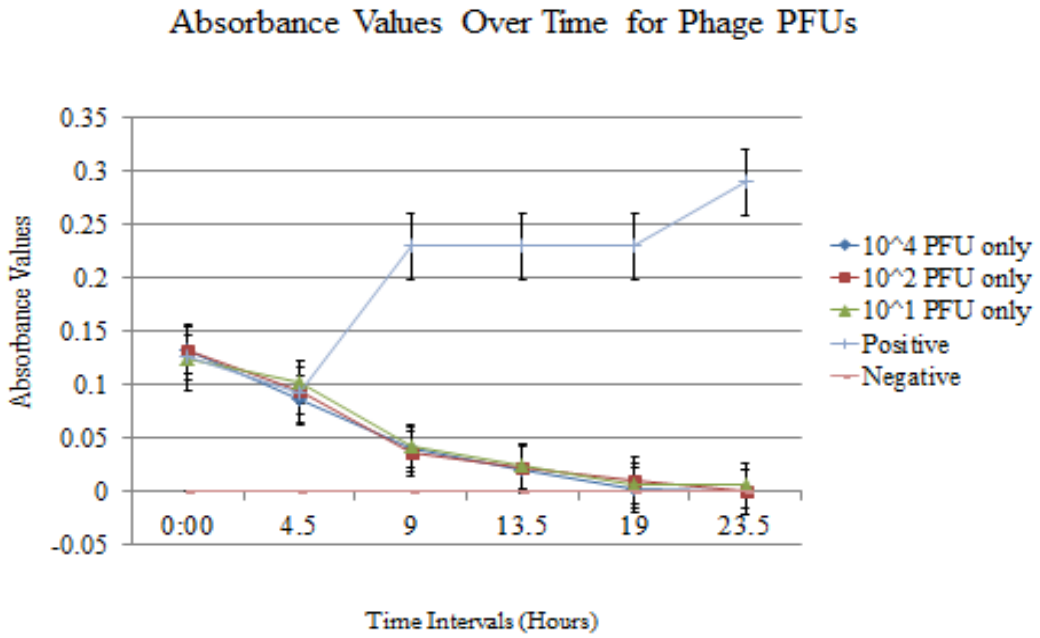

Figure 19: Absorbance values over 4.5 hour intervals for bacteriophage only, with PFUs of $1x10^1$, $1x10^2$, $1x10^4$ per mL.

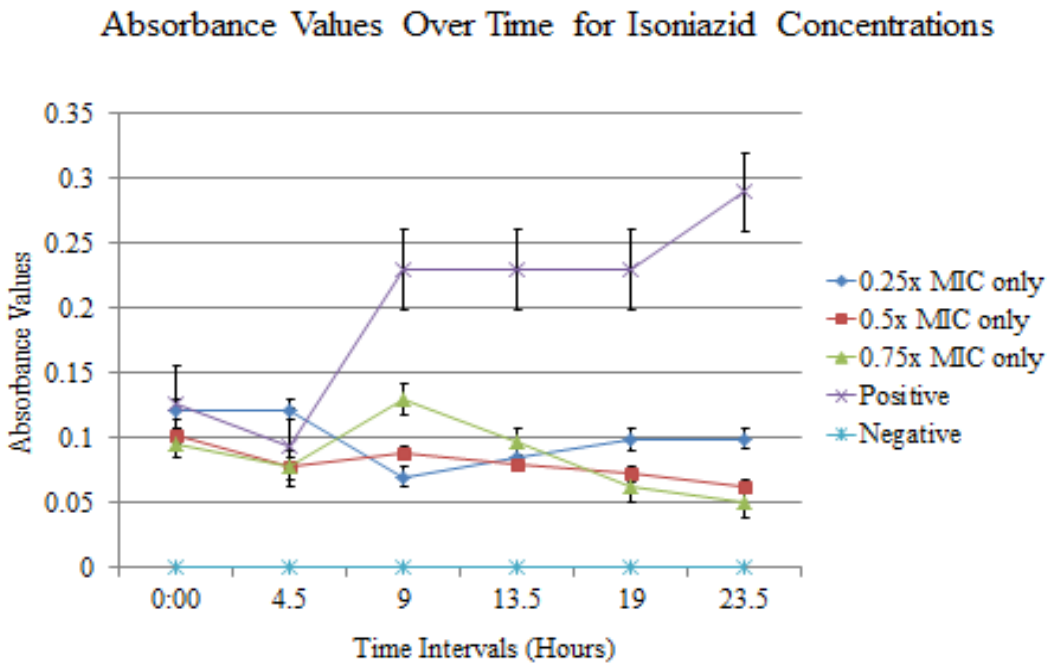

Figure 20: Absorbance values over 4.5 hour intervals for isoniazid only, with concentrations of 0.25x, 0.50x, 0.75x the MIC.