4-22-2016

Bacteriophages that can Lyse Antibiotic Resistant Bacteria

Michael Monaco
Western Michigan University, monacomk@gmail.com

Lysing Antibiotic Resistant Bacteria with Bacteriophages

Michael Monaco

Mentors: Dr. Silvia Rossbach & Dr. Karim Essani

Undergraduate Honors Thesis

Lee Honors College

Western Michigan University

Department of Biological Sciences

1903 W Michigan Ave, Kalamazoo, MI 49008

Abstract:

A *Klebsiella pneumoniae* bacteriophage was isolated from waste water and purified. This bacteriophage was imaged with transmission electron microscopy and tested with selected antibiotics for effectiveness in reducing the growth of *K. pneumoniae*. The phage was shown to reduce the growth of *K. pneumoniae* when used together with an antibiotic that the bacterium was susceptible to.

Introduction

Antibiotic resistant bacteria have become a much bigger issue in medicine than they were a few decades ago. The rise in untreatable infections that were previously curable has become a serious issue. To combat this problem, bacteriophages can be used as an alternate treatment option.

Bacteriophages are viruses that infect bacterial cells (1). The term bacteriophage was coined by Felix d'Herelle when he discovered them in 1917 (2). He tested them initially on cows and chickens and results were successful (3). He found that these "bacteria eaters" could be isolated, purified, and administered to humans as well to treat bacterial diseases like dysentery and gangrene. Phage therapy was used in a widespread manner for a number of years from the beginning of the 20s and into the 50s (3). However, with the discovery of penicillin and other antibiotics, bacteriophages became an afterthought.

Antibiotics are a very broad category of substances that are produced by one organism to kill another (4). Bacteria and fungi produce many different kinds of antibiotics that destroy different parts of the bacterial cell. Antibiotics therefore, are used in the treatment of all types of bacterial infections. Treatments are changing though because bacteria are evolving into strains like the carbapanem resistant *Klebsiella pneumoniae* (KPCs). This means a class of antibiotics typically used to fight Gram-negative bacteria, the carbapenems, are entirely ineffective against these strains of *K. pneumoniae* (5). This presents many issues for treatment when the last line of defense against an

infection no longer works. With some bacteria now having resistance to almost all known antibiotics, bacteriophages once again are being looked to for treatments.

Bacteriophages work to kill bacteria by immediate lysis. This is the process of how phages will initiate entry into a bacterium, get their DNA or RNA transcribed, and then take over the cellular machinery within the bacteria to produce hundreds of copies of the original phage. Once the phages are assembled, they will trigger the lysis of the bacterial cell, releasing all of the phages into the environment (1).

Some phages are also capable of entering the lysogenic cycle. These so called temperate phages have the ability to integrate their genetic material into the bacterial chromosome. At this point, the genetic material is considered to be a prophage and it can replicate with the bacterial chromosome and be copied into daughter cells. It will sit dormant until it is induced to enter the lytic cycle either through cellular stresses that might kill off the bacteria, such as chemical factors or UV light (6).

As mentioned earlier, phage therapy is the use of bacteriophages to treat bacterial infections within humans. Now more than ever, phage therapy holds potential to fight back against some deadly bacteria and save lives in the process. The benefits of phage therapy far outweigh the disadvantages of using bacteriophages in the human body. It is easy to gather and begin isolating new phages and it does not take much longer than a few weeks to have a new purified phage ready (3). Nevertheless, since humans have an antibody mediated response against viruses, using the same phage more than once is not going to work, unless the phage is encapsulated in a liposome (7)(8). This means research will always be active in the field and new phages will continue to be isolated. Another advantage is that phages can mutate along with bacteria, so every time a defense is

made against viral attack by the bacteria, a phage can evolve to find a way around it (1).

Best of all, phage therapy treatments should only need to be administered once; since phages can reproduce themselves, once they arrive at the infection site they should be capable of working with the immune system to wipe out the infection before being killed off themselves. The treatments can also be administered intravenously or swallowed as a pill (2). Furthermore, the antibody mediated response against the phages will likely be through IgG or IgM antibodies (9). Since IgE antibodies are responsible for allergic reactions within humans, they need to be activated in order to cause an allergic response. If IgG and IgM antibodies are instead being activated, then an allergic response will not occur. The diversity in treatment form and the inability of the phage to cause an allergic reaction spells great promise for phage therapy.

I became interested in how phage therapy might be affected by the interaction between antibiotics and bacteriophages. Through further investigation into the subject, I came across a study done by Torres-Barceló and her associates (10). In this study, their experiment showed that when adding a single dose of bacteriophage compared to a single dose of antibiotic, both would initially work, but the populations of *Pseudomonas aeruginosa* would rebound. However, when they added the two together, the population density would decrease more than if either of the components were administered individually and the population would not rebound. It is also interesting to note that when the antibiotic was added with a 12 hour delay, the bacterial population showed the largest decline. Based on this information I hypothesized that if I used an antibiotic that was ineffective on its own against *K. pneumoniae* (ampicillin) and combined it with a bacteriophage, I could decrease the growth of the bacteria more than with either of the

two individually. This would effectively render the antibiotic useable against the bacteria. I also reasoned that if the bacteriophage could provide pressures against the bacteria to divert its defenses against it, the antibiotic would have an easier time killing the bacteria.

Materials & Methods

The phages were gathered from samples of primary influent water taken from the Kalamazoo waste water treatment plant (May, 2015). From those, the samples were passed through a 22 micron filter attached to a syringe and were plated with 3 mL of lambda soft agar (10 g/L tryptone, 2.5 g/L NaCl, and 6 g/L Bacto-Agar)(11) onto various species of bacteria in their specific media. *K. pneumoniae* was selected as the bacterium for this particular project and LB (5 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract) was added as the medium. When solid medium was required, 15 g/L of Bacto-agar was added (11).

Phage Lysate Preparation

After the plates were incubated for 24 hours at 37 °C, individual plaques (clearings in the bacterial lawn indicative of cellular lysis) of different visual morphology were picked using a pasteur pipette. These plaques were then dropped into 1 mL of *K. pneumoniae* culture and incubated for 30 minutes at 37 °C before being plated with 3 mL of the lambda soft agar and incubated once more for 24 hours. The top agar was scraped off these plates with a spatula that was soaked in ethanol and flamed. This top agar was placed into a corex tube with 2.5 mL of SM buffer (0.1 M NaCl, 0.02 M tris at pH 7.5, 0.01 M $MgSO_4$, and 0.01% gelatin per 100 mL)(11) and a drop of chloroform. This tube was then centrifuged at 7,000 rpm, 4 °C, for 15 minutes. The supernatant was removed

using a sterile pipette and this created the first lysate. This lysate was stored in a glass vial at 4 °C with a few drops of chloroform until serial dilutions were ready to be made.

Serial Dilutions

From the lysate 10 μL was removed with a pipette and added to 990 μL of SM buffer in a micro centrifuge tube. This was vortexed for approximately 10 seconds and then 100 μL of this solution were added to a new micro centrifuge tube with 900 μL of SM buffer in it. This process was repeated until 5 or 6 dilutions were made. From these dilutions, 100 μL of solution were removed with a sterile pipette for each dilution being plated. The solution was added to 1 mL of the *K. pneumoniae* broth culture in a test tube and they were incubated together for 30 minutes at 37 °C. Three mL of the lambda soft agar was added to the tubes and the solution was poured onto LB plates. A negative control of only the broth culture and soft agar was used each time to ensure the bacteria were growing properly and that no other phages contaminated the bacteria. This process was repeated until the lysate was deemed pure. This was typically after 4-5 rounds of plaque picking; meaning the final lysate was typically the 5th or 6th lysate made overall.

TEM Preparation

Once a "pure" lysate was obtained, the process for imaging began. For this, the titer of the bacteriophage needed to be such that confluent lysis occurred; the plate would appear to have a loose web like structure in the soft agar from all that was remaining of the bacterial lawn. For this project, the 10^{-3} dilution was what created the confluent lysis I needed. This plate was taken and 5 mL of phage buffer (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, and 0.24 g/L KH_2PO_4)(J. Stukey, Hope College personal communications) was added to the plate. It was left to sit for approximately 4 hours. Af-

terwards, a 5 mL syringe was used to collect any buffer that remained. A 22 micron filter was attached to the syringe and the buffer was passed through into a 15 mL conical test tube. Phage buffer (1.4 mL) was then removed and placed into a microcentrifuge tube and centrifuged for 1 hour at 4 °C and 10,000 x g. Once the centrifugation was completed, most of the supernatant was removed (about 50 µL was left with the pellet) and 75 µL of fresh phage buffer was layered onto the pellet. This tube was stored overnight at 4 °C.

The final step in the imaging preparation process was done in WMU's Imaging Center. Once there, 10 µL of the buffer was removed from the micro centrifuge tube and placed onto an imaging grid. The buffer sat on the grid for 2 minutes and then excess buffer was dabbed off with filter paper. The grid was then rinsed with 10 µL of sterile water which also sat on the grid for 2 minutes before excess was removed. Finally, 10 µL of 1% uranyl acetate was added to the grid and allowed to stain for 2 minutes. The excess uranyl acetate was dabbed off and the sample was imaged with the transmission electron microscope (model JEM-1230, JEOL, Peabody, MA).

Antibiotic Susceptibility Testing

The broth culture of *K. pneumoniae* and was spread evenly across a LB plate with a plastic spreader. Antibiotic discs were placed on the plates and incubated at 37 °C for 24 hours. After incubation was completed, the zones of inhibition were measured with a ruler. For the minimal inhibitory concentration (MIC) determination, 100 mg of each antibiotic were dissolved in 1 mL of DMSO. Fifty mL plastic test tubes were then filled with 5 mL of LB broth (1st tube had 10 mL) and 12.8 µL of antibiotic solution was added to the first test tube giving it a concentration of 128 µg/mL. This tube was then vortexed

for 10 seconds on a low setting and 5 mL of the solution was removed and added to the next tube. This halved the concentration of the antibiotic to 64 μg/mL in the second tube. This process was repeated until the concentration in the final test tube was 1 μg/mL. The final step was to add 50 μL of *K. pneumoniae* broth culture to each tube, including a negative control which only had 5 mL LB broth in it. These tubes were incubated in a 37 °C shaker for a minimum of 18 hours. Once incubation was completed, whichever tube with the lowest concentration of antibiotic to not show turbidity was considered to represent the MIC.

Antibiotic and Phage Testing

This testing was done by setting up six controls and six test plates. All plating methods were the same as before, but in addition, antibiotics were added at their measured MICs. As controls, one plate of only *K. pneumoniae* was made and two plates of only bacteriophage at concentrations of 10^{-3} and 10^{-2} were made. These concentrations were selected because at the lower concentration, there was a web-like pattern of cell lysis and at the higher one, individual colonies would survive. Finally, three plates of each antibiotic at its MIC were combined with *K. pneumoniae* to become the last three controls.

Results

A phage I had named *K. pneumoniae* 2B was chosen for the experiments. This was because the plaques were abundant and would appear even at low concentrations (10^{-7}). The plaques appeared as small, transparent circles with no rings (Fig. 1A). At the concentration of 10^{-3}, the bacterial lawn would exhibit a web-like pattern of cell lysis and most of the bacterial lawn was destroyed (Fig. 1B). This phage also exhibited very inter-

esting behavior at the highest concentrations (10^{-2} and above). Rather than the bacterial lawn becoming lysed entirely, there would be individual colonies surviving (Fig. 1C).

To obtain images of the phage, a transmission electron microscope was used. Pictures were taken at three different magnifications: 100,000x, 150,000x, and 200,000x. Only the highest magnification allowed for clear depictions of the viral structures (Fig. 2). The phage exhibited a length of approximately 220 nm. There were indications of a base plate, sheath, tail fibers, and a well defined head. The phage sample however, due to the presence of a very small spherical object in the sample, may not have been pure (yellow arrow in Fig. 2).

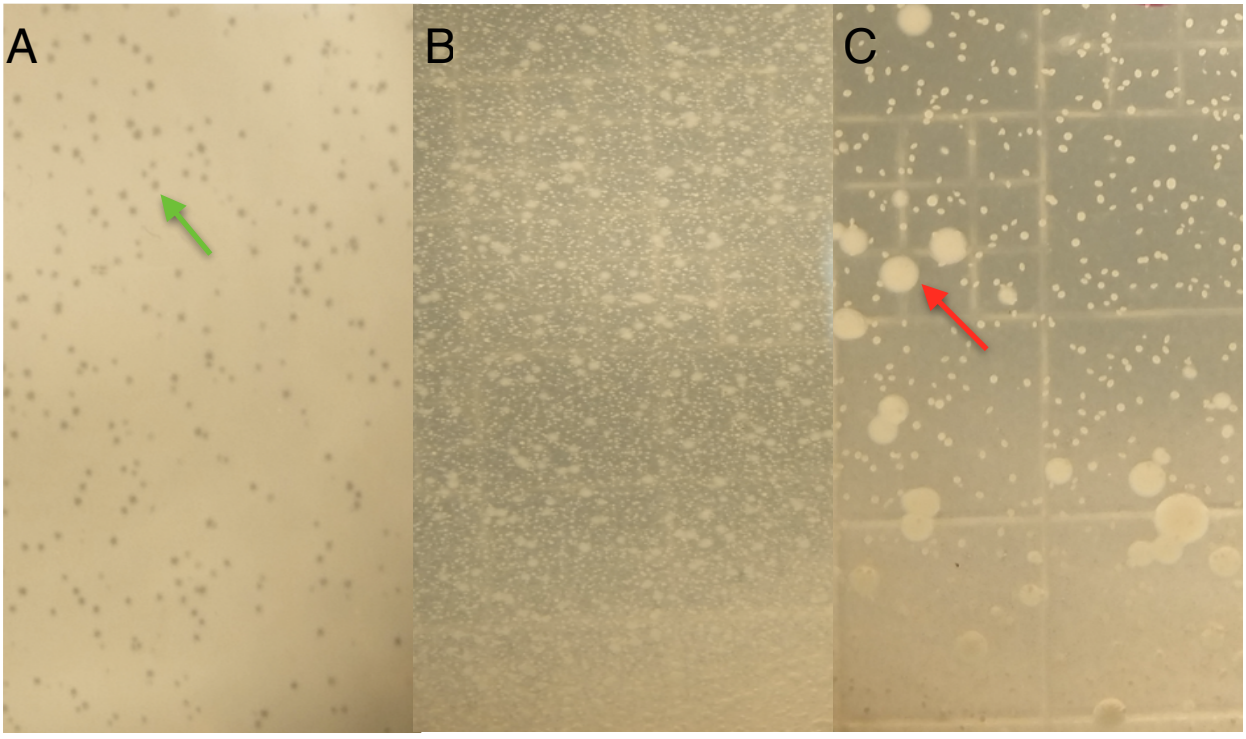

Fig. 1: Photographic images of plates containing *K. pneumoniae* and phage 2B. A shows phage 2B at a concentration of 10^{-4}. The green arrow on A points to one of over one hundred plaques created by the phage under normal circumstances. B shows an example of phage 2B at 10^{-3} where the bacterial lawn exhibits a weblike pattern of cell lysis. C shows the phage at a concentration of 10^{-1}. It shows an example of potential lysogenic behavior exhibited by phage 2B when at concentrations of 10^{-2} and above. The red arrow shows one of the many individual *K. pneumoniae* colonies that survived lysis. Each large square in C is 1 cm across.

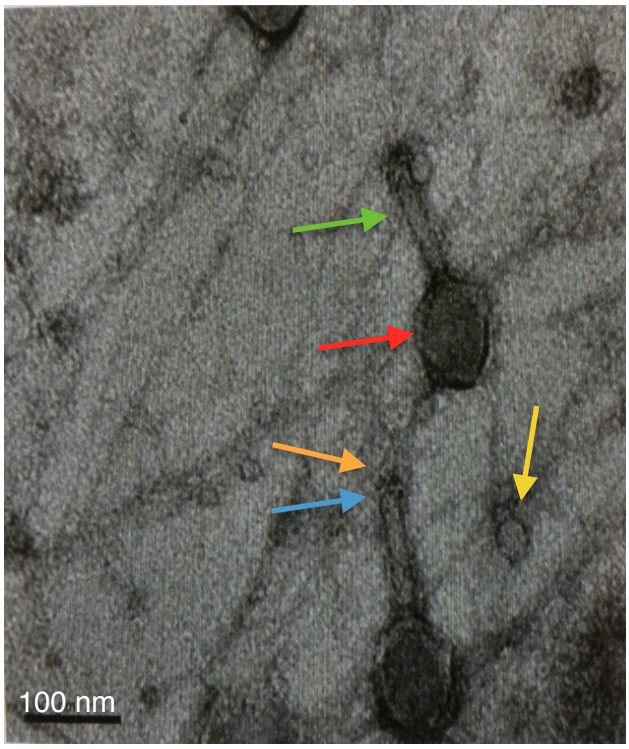

Fig. 2: An electron microscopic image of phage 2B at 200,000x zoom. The red arrow points to the head of the phage, the green arrow to the sheath of the phage, the blue arrow to the base plate, and the orange arrow to the tail fibers of the phage. The yellow arrow points to a possible contaminant phage or artifact of the preparation. The black bar at the bottom represents 100 nm.

Table 1
Antibiotic susceptibility testing with *K. pneumoniae*

Antibiotic	Zone of Inhibition (mm)[a]	Susceptibility[b]	MIC (µg/mL)
Ampicillin	0	Resistant	16
Tetracycline	15	Intermediate	0.5
Streptomycin	22	Susceptible	1

[a] All zone of inhibition measurements include 6 mm for the disc. [b] Susceptibility results were determined using a BD BBL chart (12).

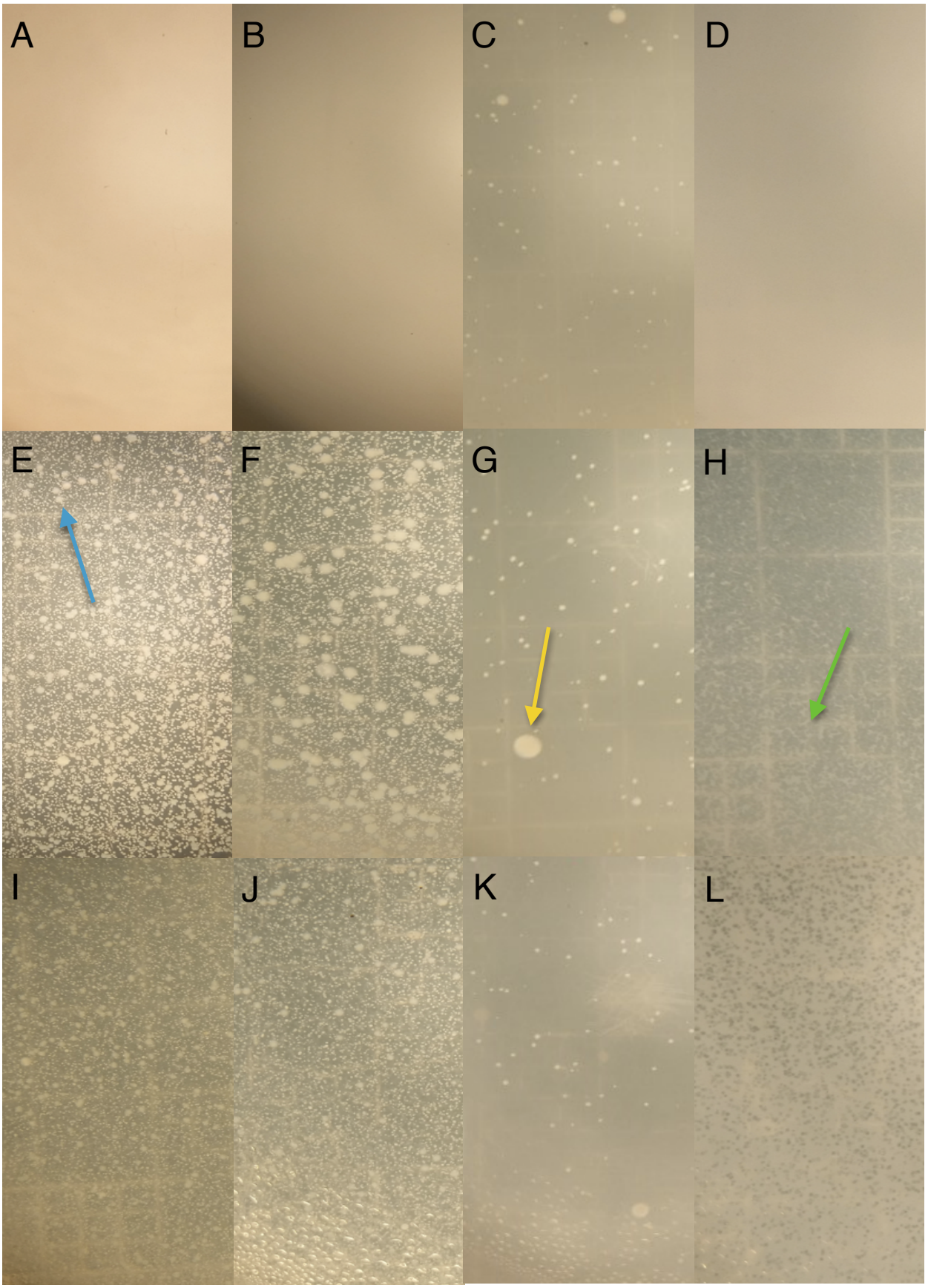
A
B
C
D
E
F
G
H
I
J
K
L

Fig. 4: Photographic images showing results of the antibiotic/bacteriophage experiment. A shows the *K. pneumoniae* control. B, C, and D show the ampicillin, tetracycline, and streptomycin controls, respectively. E shows the phage 2B control at 10^{-2}. The blue arrow on E is another example of the individual colonies that would form when phage 2B was at high concentrations. F, G, and H show ampicillin, tetracycline, and streptomycin combined with phage 2B at 10^{-2} respectively. The yellow arrow on G shows that individual colonies appearing with the absence of a bacterial lawn. The green arrow on H points to the lack of individual colony formations when streptomycin was combined with phage 2B. I shows the phage 2B control at 10^{-3}. J, K, and L show ampicillin, tetracycline, and streptomycin combined with phage 2B at 10^{-3} respectively.

After imaging phage 2B, I decided to test the phage's ability to lyse *K. pneumoniae* when combined with antibiotics of various effectiveness. To determine this, a *K. pneumoniae* plate was made with LB medium and a disc assay was performed. I selected three antibiotics: ampicillin because *K. pneumoniae* was resistant to it, tetracycline, because according to a BD BBL chart, a 15 mm zone of inhibition (ZoI) was considered intermediate resistance for *Klebsiella* species, and streptomycin, because a 22 mm ZoI was considered to be susceptible. After these antibiotics were selected, the MICs for each antibiotic were determined. The MIC for ampicillin was determined to be 16 μg/mL, for tetracycline, it had inhibited growth at all concentrations up to 0.5 μg/mL, and streptomycin's MIC was determined to be 1 μg/mL (Table 1). These results did not necessarily agree with each other. One would think that if tetracycline was only partially effective against *K. pneumoniae*, then the MIC would be in-between streptomycin and ampicillin. Instead, the dissolved concentration of tetracycline was the best at preventing bacterial growth out of the three antibiotics.

The primary experiment combining phage 2B and antibiotics was performed as described in the methods section. First, the results with ampicillin were not supporting my hypothesis, they were actually rejecting it. Not only was ampicillin unable to prevent growth at the MIC by itself, when combined with the phage, the control plates looked

hardly different than the combination plates (Fig. 4B, F, & J). The only difference between the ampicillin results and the controls were fewer and slightly larger colonies on the ampicillin plates. When looking at the tetracycline containing plates, tetracycline had prevented a bacterial lawn from ever developing which then prevented any plaques from appearing (Fig. 4C, G, & K). Only dispersed colonies were observed in all cases. Finally, when looking at streptomycin, which was effective against *K. pneumoniae* in the disc assay, the plates showed much more promising results (Fig. 4D, H, & L). Not only was the bacterial population reduced compared to the controls, but also the individual colonies that were present on the control plates were not observed on the streptomycin plates (Fig. 4H).

Discussion

The presence of individual colonies at high phage concentrations is worth discussing. There are two possibilities that could explain why the colonies persisted: either the phage was temperate and could enter the lysogenic cycle or the bacteria were evolving resistance to the phage. The problem with the latter is that if the bacteria were evolving resistance, individual colonies would not appear at only the highest concentrations. On the contrary though, the bacteria could very well have evolved to mutate the receptor that the phage binds to. This would prevent the phage from being able to enter and lyse the bacteria. It would be also be difficult to determine resistance at low phage concentrations because if only a few plaques exist, you could not determine if this was due to the phages inability to lyse the bacteria or if it was due to the low phage concentration itself. A more definite way of determining temperateness would be to expose a high concentration phage 2B plate with individual colonies to UV light and see if that caused

those colonies to become lysed. If they were lysed, I would say I that phage 2B is a temperate phage, but if the bacteria were not lysed, I would say that the bacteria had acquired resistance.

When looking at the transmission electron microscopy images, phage 2B showed characteristics that resembled a T4 phage (13). It was approximately 220 nm in length and had clearly defined structures like the head, sheath, and tail fibers. Even though T4 phages are exclusively lytic and belong to a family of *E. coli* phages (1)(13), phage 2B's structure more closely resembled the T4 phage than lambda, which is a typical lysogenic phage. This is because the lambda phage typically has a longer tail than its head and it does not have a defined base plate or tail fibers. Similarities between the structures, however, do not necessarily point to similarities in function. It could be mere coincidence that phage 2B appears to look like a known lytic phage.

Another issue was that there appeared to be a spherical artifact in the microscopic sample. It could be due to the presence of another phage, or perhaps it is just an artifact of the preparation. If the sample happened to actually contain more than one phage and the plaques were merely morphologically identical, then the only way to determine purity would be to plate again, pick an individual plaque and re-increase the titer for imaging. If the spherical structure is not present, one could be more confident the sample was pure, or if there is nothing except the spherical objects, the second phage was part of the original lysate.

The results of the antibiotic and bacteriophage experiment appeared to disprove my hypothesis, especially so with ampicillin. The only other way that I could try to support my hypothesis, in regards to using an ineffective antibiotic, would be to perform the

entire experiment in liquid. This would enable me to add the antibiotic at the 12 hour point which Torres-Barceló et al. showed increased the effectiveness of the phage/antibiotic combination (10). The way my experiment was set up, once the soft agar is added to the plate, it would dry in about 5 minutes and nothing more could be added. The entirely liquid version of this experiment might complicate how results should be interpreted though. If liquid results showed that ampicillin can actually help reduce *K. pneumoniae* population density, a wider range of antibiotics might be usable.

On the streptomycin plates, both the individual colonies would not be observed on the high phage concentration plate and the overall bacterial population was reduced compared to either of the controls. This supported the notion that using antibiotics together with bacteriophages can be more effective than either of them alone, but, the antibiotic needed to be effective by itself to improve cellular lysis when combined with the phage. This experiment should be repeated as designed though. If the results on the second or even third replication match the initial results, I could further strengthen the hypothesis that phages and antibiotics work better together. It would be clear that using an antibiotic that the bacterium is known to be susceptible to is the best option when considering what to combine with an isolated phage.

Conclusions

Based on the data that were gathered in this experiment, the results support the fact that bacteriophages on their own will reduce bacterial populations. With the correct initial concentration, a bacteriophage has the potential to eliminate an infection entirely. It was also shown that when a bacteriophage is combined with an antibiotic the bacteria is susceptible to, the density of the bacterial population is reduced further than

when either was used individually. These data provide enough evidence to research the topic further for effectiveness within the human body and when the best time to administer the antibiotic would be. The potential for phage therapy to become a life saving treatment for people suffering from bacterial infections acquired in hospital or in the community where the bacteria have been exposed to antibiotics is tremendous. Hopefully the scientific community within the United States will begin researching these phages again. Perhaps one day, bacterial infections from strains like methicillin resistant *Staphylococcus aureus* (MRSA) or KPC will eventually be curable through the use of phage therapy.

Acknowledgements

I would like to thank the Lee Honors College first and foremost for allowing me to use this research for my Senior Thesis. I would also like to thank my mentors Dr. Silvia Rossbach and Dr. Karim Essani for providing invaluable support and guidance to address any questions I had or with any problems I encountered along the way. I want to thank Carol Beaver for staying in the lab until late in the day in order to allow me to use the centrifuge and for being available to help whenever I needed it. I would like to thank Torin Kulhanek and Dr. Robert Eversole for training me to use the TEM in the Imaging Center and for being available to provide materials and helping me with the imaging process. I would like to thank the Undergraduate Research and Creative Activities Award committee for giving me a grant to help fund this research. I also want to thank Zach Schmidtke and Ian Crumm for their assistance on this project as my lab partners. I am very grateful for their contributions. Finally, I would like to thank my family for providing me with the opportunity to attend WMU and for being understanding and sup-

portive when I had to stay at school for extended periods of time to work in the lab at midnight on the weekends.

References

1. Willey, J. M., Sherwood, L., Woolverton, C. J., and Prescott, L. M. *Prescott's Microbiology*. New York: McGraw-Hill, 2011. Print.
2. Carlton, R. M., *"Phage Therapy: Past History and Future Prospects."*, Archivum Immunologiae et Therapiae Experimentalis, 1999, 47, 267–274
3. http://phages.org/
4. http://medical-dictionary.thefreedictionary.com/antibiotic
5. http://www.cdc.gov/HAI/organisms/klebsiella/klebsiella.html

6. Lorenz, N., Reiger, M., Toro-Nahuelpan, M., Brachmann, A., Poettinger, L., Plener, L., Lassak, J., and Jung, K. (2016) *Identification and Initial Characterization of Prophages in Vibrio campbellii*. PLoS ONE 11(5): e0156010. doi:10.1371/journal.pone.0156010

7. Dörner, T. and Rodbruch, A., "*Antibodies and B Cell Memory in Viral Immunity*." Immunity, Volume 27, Issue 3, 384 - 392 (2007)

8. Singla, S., Harjai, K., Katare, O.P., and Chhibber, S. (2016) *Encapsulation of Bacteriophage in Liposome Accentuates Its Entry in to Macrophage and Shields It from Neutralizing Antibodies*. PLoS ONE 11(4): e0153777. doi:10.1371/journal.pone.0153777

9. Hodyra-Stefaniak, K., Miernikiewicz, P., Drapala, J., Drab, M., Jonczyk-Matysiak, E., Lecion, D., Kaźmierczak, Z., Beta, W., Majewska, J., Harhala, M., Bubak, B., Kłopot, A., Górski, A., and Dąbrowska, K. (2015). *Mammalian Host-Versus-Phage immune response determines phage fate in vivo*. Sci. Rep. 5:14802. doi: 10.1038/srep14802

10. Torres-Barceló, C., Arias-Sánchez, F. I., Vasse, M., Ramsayer, M., Kaltz, O., and Hochberg, M. E., "*A Window of Opportunity to Control the Bacterial Pathogen Pseudomonas Aeruginosa Combining Antibiotics and Phages*." PLoS ONE. 9.9 (2014)

11. de Bruijn, F. J. and Rossbach, S., 1990. Transposon Mutagenesis, p. 387-405. *In* Gerhardt, P., Murray, R. G. E., Wood. W. A., and Krieg. N. R., *Methods for General and Molecular Bacteriology*. Washington, D.C.: American Society for Microbiology, 1994. Print.

12. https://www.bd.com/ds/technicalCenter/inserts/8840621(201107).pdf

13. Yap, M. L., & Rossmann, M. G. (2014). *Structure and function of bacteriophage T4*. Future Microbiology, 9, 1319–1327. http://doi.org/10.2217/fmb.14.91