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Isolation of a Mycobacterium Virus with the Infectivity Rate Tested at Various Temperatures

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ABSTRACT

Isolation techniques developed by the HHMI and the SEA-PHAGES program were used to obtain a bacteriophage from a mixture of loamy clay soil. The sample was retrieved from a forest edge in central Kentucky. The isolate produced large lytic plaques on a lawn of the bacterium *Mycobacterium smegmatis*. The bacteriophage was purified, amplified, and analyzed using restriction digest analysis with visualization by gel electrophoresis. Restriction digest analysis suggests the virus belongs to a group known as "A". Mycobacteriophages are placed into distinct groups based on DNA sequences comparison. The next step in the procedure will be to document with an electron micrographic image. The virus will then be sequenced, so the genome can be annotated. The virus will be able to be further characterized in its cluster during the annotation process. Future studies include testing rates of infectivity of at various temperatures. It is my goal that these studies will allow the determination of a novel bacteriophage.

INTRODUCTION

Viruses are more abundant than any other organism on Earth, including bacteria. For every type of organism such as animals, plants, bacteria, and fungi there is at least one virus to infect the cell. The most abundant type of virus is a bacteriophage (1). A bacteriophage infects bacterial cells. Even though viruses are more common than any other organism, they are not considered to be alive because in order for a virus to reproduce it must be inside a host cell. Viruses are not able to replicate outside of a host cell, and therefore are not considered to be living. A virus consists of some type of genetic material, DNA or RNA, and some kind of protein coat. Viruses have adapted many different ways to infect host cells, so they can reproduce and infect other cells. Many viruses have a protein coat that has the correct surface proteins to bind to

the surface receptors on the host cell. The virus can also mimic another cell in the host, this is if the cell is eukaryotic, that interacts with the cell the virus wants to infect. Another way a virus successfully infects the host cell is to enclose itself in a lipid membrane from the host cell that it infected previously. When the virus is able to do this it is virtually undetectable by the host cell. Viruses must have the correct proteins exposed if they want to infect a host cell. If the surface receptors on the host cell and virus do not match, the virus will not be able to infect the host cell because the virus will not be able to bind to the host cell. This is why viruses can only attack certain host cells and not every type of cell that exists.

The Howard Hughes Medical Institute (HHMI) is very interested in research that relates to healthcare. HHMI collaborated with the University of Pittsburgh to make a program to that would help get high school students interested in science (2). HHMI called the program SEA-PHAGES which stands for Science Education Alliance Phage Hunters Advancing Genomics and Evolutionary Science (2). The purpose of the program is to find viruses in the soil that can infect *Mycobacterium smegmatis*. HHMI has a goal of finding a virus that can infect *Mycobacterium tuberculosis* because there are strains of tuberculosis that are becoming antibiotic resistant, and it is a growing problem (2). They can accomplish this through the SEA-PHAGES program because *Mycobacterium smegmatis* is in the same family of bacteria as *Mycobacterium tuberculosis*.

The goal of this research is to test if there are differences in the rate of virus infectivity at various temperatures. In order to accomplish this, a Mycobacterium virus is isolated from soil. The infectivity rate of the virus is then tested at 37^0C and 30^0C. The hypothesis for the experiment is that there will be a difference of the rate of infectivity at these temperatures. The thought is that the difference will occur because different surface proteins will be expressed at different temperatures.

METHODS

Methods were adapted from Phagesdb.org. (3)

Soil Collection

Soil is collected from Quail Chase Golf Course in Louisville, Kentucky in three different locations on the course. Three 50 mL conical tubes are used to collect soil from each location totaling nine conical tubes. Each 50 mL tube has about 50 grams of soil.

Soil Screening

After the soil is collected, 10 grams of soil is mixed with 50 mL of phage buffer containing 500 μL of 1mM $CaCl_2$ and 5 mL of *Mycobacterium smegmatis*. The mixture is incubated at 37^0C with shaking at 225 rpm for 48 hours.

Testing for Virus

Once the soil has shaken for two days in the incubator, it is time to test to see if there is virus in the sample. This can be done in two different ways. The first way is to perform a spot test, which is the quickest way to see if there is phage in the sample. In this test 500 μL of *Mycobacterium smegmatis* are mixed with 4.5 mL of top agar, pipetted onto a 7H10 plate with cycloheximide (CHX) and carbenicillin (CB). The top agar is allowed to solidify for approximately 10 minutes. Next, 10 μL of the sample are gently pipetted onto the plate. It is best to draw a grid on the plate to avoid confusion if plating different samples on the same plate. The other method of testing for phage involves using top agar and freshly grown *Mycobacterium smegmatis*. With this test, 500 μL of *M. smegmatis* are incubated with 50 μL

of the sample (which hopefully contains virus) for at least 20 minutes in a small test tube. After 20 minutes 4.5 mL of top agar containing 1mM $CaCl_2$ is mixed with the sample by a pipette totaling 5 mL. This is then gently pipetted onto a 7H10 plate containing cycloheximide (CHX) and carbenicillin (CB). Next, wait approximately 10 minutes for the top agar to solidify before turning the plate upside down to place in the incubator. The plate is allowed to grow for two days in an incubator at 37^0C. A negative control is used with each of these methods, and it can be phage buffer or water.

Virus Purification

The next step is to purify the virus in the sample to make sure that there is just one virus. In order to accomplish this, serial dilutions of the sample must be made. A plaque is picked from the plate that is isolated, so it is known that there is only type of virus from this point on. From this, the plaque is added to 100 μL of phage buffer with 1mM $CaCl_2$. The serial dilutions then take place with 10 μL of the mixture being added to 90 μL of phage buffer. This is the 10^{-1} dilution. This is repeated to the 10^{-4} dilution. These dilutions are then plated on 7H10 plates with CHX and CB in the same manner as the second method for testing for virus. The plates are incubated at 37^0C for two days. Three rounds of this purification are necessary to make sure that there is only one virus.

Testing the Rate of Infectivity

Once a pure sample of virus has been obtained, the infectivity rate can be tested at 37^0C, which is the optimum temperature for *M. smegmatis* to grow, and 30^0C. The virus is tested at each temperature. This method uses the same procedure outlined in testing for virus. Any

differences will be visualized by looking at the plates after 48 hours of growth. A picture of each plate is taken to record the differences.

High Titer Lysate

Once the virus is purified a high titer lysate can be made to increase the concentration of the virus. This will help to obtain a high concentration of DNA from the virus when the DNA is extracted. The concentration of the high titer lysate needs to be at least 10^8 PFU/mL. PFU stands for plaque forming units. The high titer lysate is made by calculating the current concentration of the virus when it makes a webbing pattern on the plate. The plate will be mostly clear of bacteria, and it will have multiple overlapping plaques. This means it has gone through enough rounds of infection to have enough virus to be able to collect the virus. When the concentration of the current virus is known, the correct amount will be mixed with *Mycobacterium smegmatis* and top agar with 1 mM $CaCl_2$. Three hundred milliliters of this will be made, with 5 mL being placed on each plate. This will total 60 plates. The plates are then grown for 48 hours. Next, the plates need to be checked to make sure all of them contain the webbing pattern. After this, 5 mL of phage buffer with 1 mM $CaCl_2$ will be added to each plate. The plates will then sit for about 4 hours with occasional gentle swirling of the plates, or the plates can be placed in a cold room overnight. This allows the virus to be transferred to the phage buffer. When the phage buffer has had enough time to absorb the virus, the plates are slightly tilted to bring the liquid phage buffer with the virus to one side of the plate. The liquid is then collected using a pipette and transferred to a 50 mL conical tube. This is repeated for all of the plates. The conical tubes containing the high titer lysate are then placed in a centrifugation machine for 15 minutes at 3000 rpm to remove any bacterial or agar

debris. Next, the centrifuged lysate is filtered using a vacuum into a new 50 mL conical tube. When the filtering is complete, the resulting liquid is the high titer lysate. The high titer lysate now has to be tested to see which concentration of the virus will cause the webbing pattern to appear on the plate. This done through serial dilutions of the virus that ranges from the 10^{-1} dilution to 10^{-10} dilution.

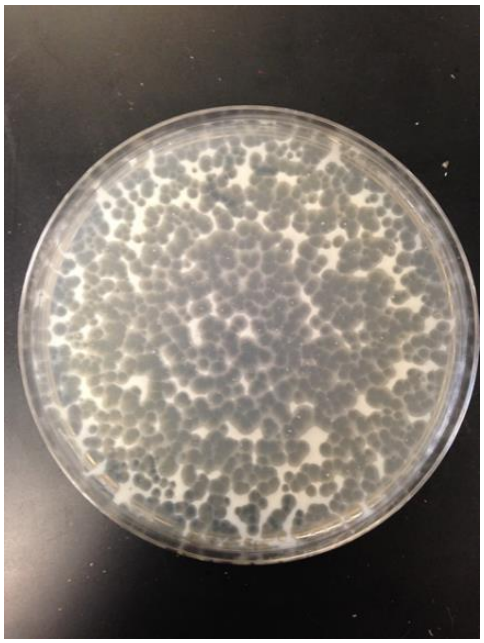

FIGURE 1: This picture shows an example of the webbing pattern on the plate that needs to be reached in order to be able to perform the high titer lysate methods.

DNA Purification and Extraction

To purify and extract the DNA, add 5 mL of the high titer lysate to a 15 mL conical tube, add 62.5 µL of 1M $MgCl_2$, and gently mix. Next add 4 µL of DNAse I (2000 U/mL) and 5 µL of RNAse A (100 mg/mL) to the mixture. Incubate at room temperature for 30 minutes after briefly vortexing the mixture. After the incubation add 200 µL of 0.5 M EDTA, 25 µL of Proteinase K (10 mg/mL), and 250 µL of 10% SDS. It is very important to add these reagents and enzymes in the order that is listed. Adding them in the wrong order can cause the extraction process to be unsuccessful. Vortex the mixture vigorously and then incubate the

mixture for an hour at 55°C. During the hour incubation, vortex the mixture twice at 20 minute intervals. Next, get ten 1.5 mL microcentrifuge tubes, and transfer 500 µL of the mixture into each tube. Using gloves and the chemical hood, add 500 µL of PCI or Phenol: Chloroform: Isoamyl alcohol (25:24:1) to each microcentrifuge tube. Limit the exposure to light for the PCI because the chemicals in the solution can become oxidized if they are exposed to light. Once the PCI is added to each tube, invert each tube several times to ensure enough mixing. Centrifuge the tubes for 5 minutes at 13,000 rpm at room temperature. After this, carefully remove the top aqueous layer that is above the white interphase in the tube, and add it to a 15 mL conical tube. It is important to make sure that none of the white interphase or the organic layer underneath are mixed with the aqueous layer because this will cause there to be phenol contamination and the DNA extraction will have to be repeated. The next step is to precipitate the DNA. To do this add 5 mL of 95% ethanol and 250 µL of 3M sodium acetate solution to the conical tube. Next, pipette equal amounts into 1.5 mL microcentrifuge tubes, and place on ice for 5 minutes. A precipitate will form. Centrifuge for 10 minutes at 13,000 rpm at room temperature. Carefully decant the tubes as so not to lose the pellets that are in the bottom of the tubes. Now, add 500 µL of 70% ethanol to wash the pellet. Centrifuge for 10 minutes at 13,000 rpm at room temperature. Decant the tubes and again be careful to not lose the pellet of DNA that has formed. Allow the pellets to air dry for 10 – 20 minutes. Add about 50 µL of deionized water to the pellet. Incubate the tubes for 10 minutes at 37°C to make sure the pellet is completely dissolved. Store the extracted DNA at 4°C for short term storage, but store the DNA at -20°C for long term storage.

Measuring the Concentration of the DNA

The concentration of the DNA is measured by using a nanodrop spectrophotometer. This machine only requires 2 µL of extracted DNA to be used. The machine is cleaned and blanked with sterile deionized water. The concentration is then measured. After the concentration is measured the machine is again cleaned with sterile deionized water.

Restriction Enzyme Digest with Visualization by Gel Electrophoresis

Once the concentration of the DNA is determined, a restriction enzyme digest can be performed. The purpose of the restriction enzyme digest is to see if the extracted viral DNA will be cut anywhere with various restriction enzymes. The restriction enzymes used in this study are BamHI, ClaI, EcoRI, HaeIII, and HindIII. These are the five standard restriction digest enzymes used to characterize viral DNA. The volume for the restriction digest is 15 µL. This means that the concentration of the DNA will determine the amount of some of the reagents that are used for the restriction digest. Obtain five microcentrifuge tubes. Add x µL of DNA, 1.5 µL of 10X buffer, 1.5 µL of 10X BSA, x µL of sterile deionized water, and 0.5 µL of the restriction enzyme. This needs to be repeated for every enzyme. It is very important to add these in the correct order because the restriction digest will not work if these are added at the wrong times. As soon as the enzyme is added, the microcentrifuge tubes need to be placed in an incubator at 37°C for 1 hour.

While the incubation is taking place, the gel for the visualization of the enzymes needs to be made. Make a 0.7% agarose gel using TAE as the buffer for the gel. To make a small gel weigh 0.35 g of agarose in an Erlenmeyer flask, add 50 mL of 1X TAE buffer, and microwave for 1 minute with a paper towel covering. When the flask as cooled off enough to

be warm to the touch, add 2 µL of ethidium bromide to the mixture. Then pour this gently into the casting tray for the gel, and place the comb to form the wells. Allow this to completely solidify.

When the restriction digest is finished, add 2 µL of a loading dye to the digest in each tube. Pipette the total amount of the mixture, 17 µL into the corresponding well on the gel. Make sure to add a molecular weight marker to be able to measure the size of the DNA bands that will form on the gel.

Run the gel for 15 minutes at 100 volts, and then turn the voltage down to 25 volts and let the gel run for another hour and 45 minutes. This allows the bands to move slowly for good separation on the gel.

When the gel is finished running, take a picture of the gel with UV light to visualize the bands formed from the restriction digest of the viral DNA. This is done by allowing the exposure time for the picture to be five seconds while the UV light is on. Be sure to wear the proper safety eyewear, as UV light can damage the eyes and skin.

Liquid Culture and Plating

In order to be able to perform all of the above methods, *Mycobacterium smegmatis* must be kept alive through liquid culture and plating. Liquid 7H9 broth with growth nutrients is used to keep liquid cultures of the bacteria alive. The bacteria is also plated onto to 7H10 plates to keep another form of pure bacteria. Both of these cultures are stored at 4°C.

RESULTS AND DISCUSSION

Isolation of Virus

The first major result is actually isolating the virus to be studied. This is done by purifying the virus by using serial dilutions. It is known the virus is pure once the plaques forming on the plates are all the same size. This is the only way to determine purity in the lab without taking electron microscope pictures of the virus to see if it is the same. It can be very clearly seen with the plaques whether or not the plaques are the same size on the plates, and this is why isolating the virus can be determined by looking at the plaques that form on the plate of bacteria.

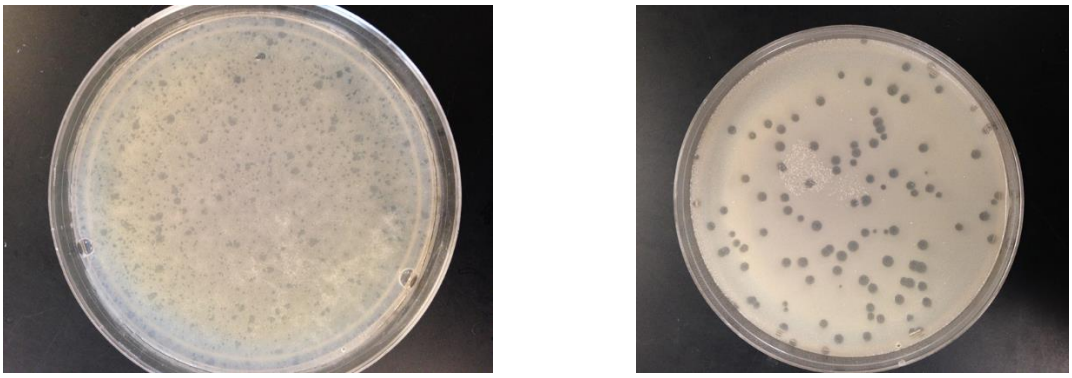

FIGURE 2: The picture of the plate on the left shows what a plate looks like before any purification takes place. There are plaques on this plate that are multiple sizes which indicates that they are different viruses. More purification of these viruses will need to be done. The picture on the right shows a plate that has gone through two rounds of virus purification. This can be seen because most of the plaques on the plate are the same size, but there are still a few plaques that are smaller. This plate just needs to go through one more round of purification to have an isolated virus.

Restriction Enzyme Digest

The restriction enzyme digest with visualization by using gel electrophoresis shows where the restriction enzymes that are used cut the viral DNA. The gel shows that the only restriction enzyme to cut the viral DNA is the restriction enzyme HaeIII. This suggests that the virus can be classified in the Cluster A family of mycobacterium viruses

because this cluster consists of viruses whose DNA only cuts at HaeIII (4). This is important because it helps to give an idea on the type of virus that has been isolated. The DNA of the virus will need to be sequenced in order to characterize the virus further.

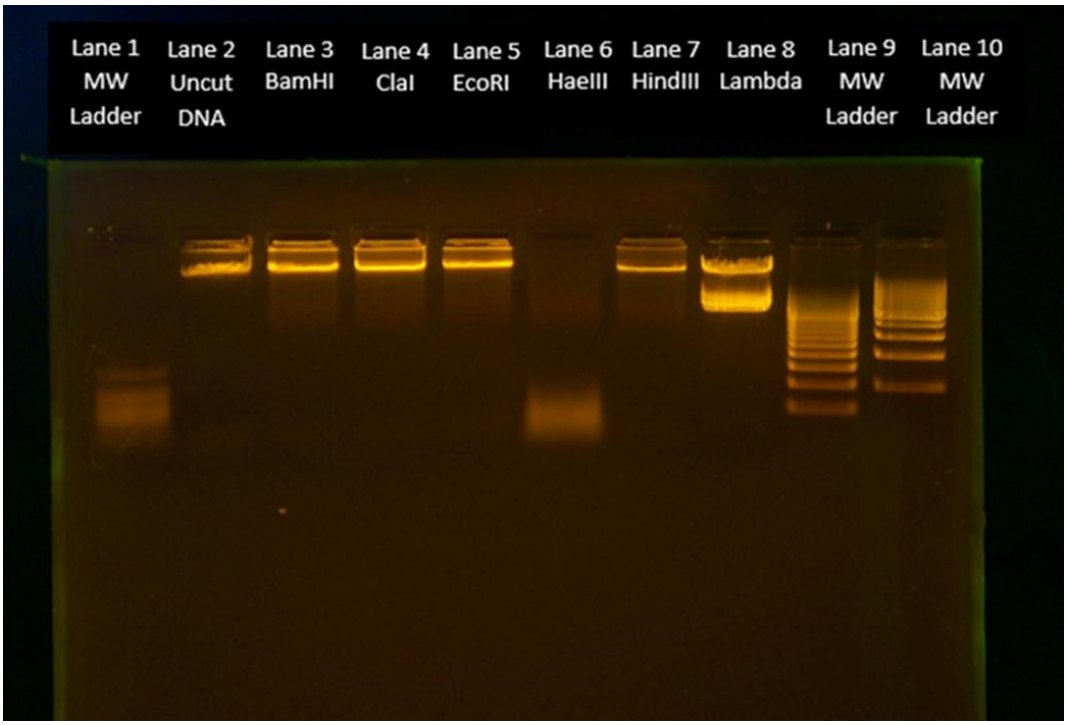

FIGURE 3: This shows the results of the restriction enzyme digest of the viral DNA with BamHI, ClaI, EcoRI, HaeIII, and HindIII. The only enzyme to cut the viral DNA is HaeIII. This known from comparing the uncut DNA in Lane 2 to Lane 6 where HaeIII is used. The DNA fragment that is cut in Lane 6 is estimated to be 500 base pairs from the molecular marker used in Lane 9. The molecular marker in Lane 9 is a 500 base pair marker, meaning the smallest measurement is 500 base pairs and it increases in increments of 500 base pairs.

Rate of Infectivity at Various Temperatures

At 37°C and 30°C, there is not a difference in the infectivity rate at the 10^{-6} dilution of the virus. However, at the 10^{-7} dilution there is a difference between the infectivity rate at 37°C and 30°C. At this dilution of the virus, the plaques that form on the plate are smaller at 30°C than they are at 37°C. This could be due to the possibility of there being different surface receptors expressed on the bacteria or the virus at various temperatures. There is a

difference in the infectivity rate at 37°C and 30°C, but the difference is also dependent on the concentration of the virus. Further research needs to be done to see why the concentration plays a role along with the temperature. More studies also need to done to measure the types of surface receptors on the bacteria and virus to see if this is affecting the infectivity rate.

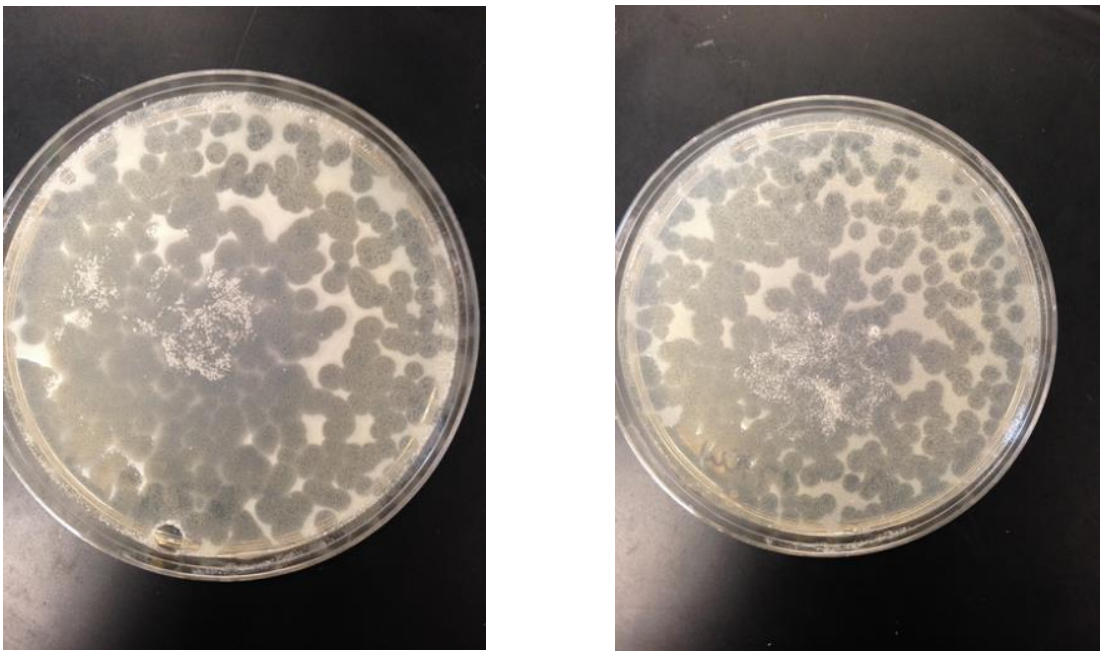

FIGURE 4: The picture on the left shows the bacteria and virus grown at 37°C with the virus at the 10^{-6} dilution. The picture of the plate on the right shows the bacteria and virus grown at 30°C with the virus at the 10^{-6} dilution. There is not a difference in plaque size at these temperatures or the 10^{-6} dilution of the virus.

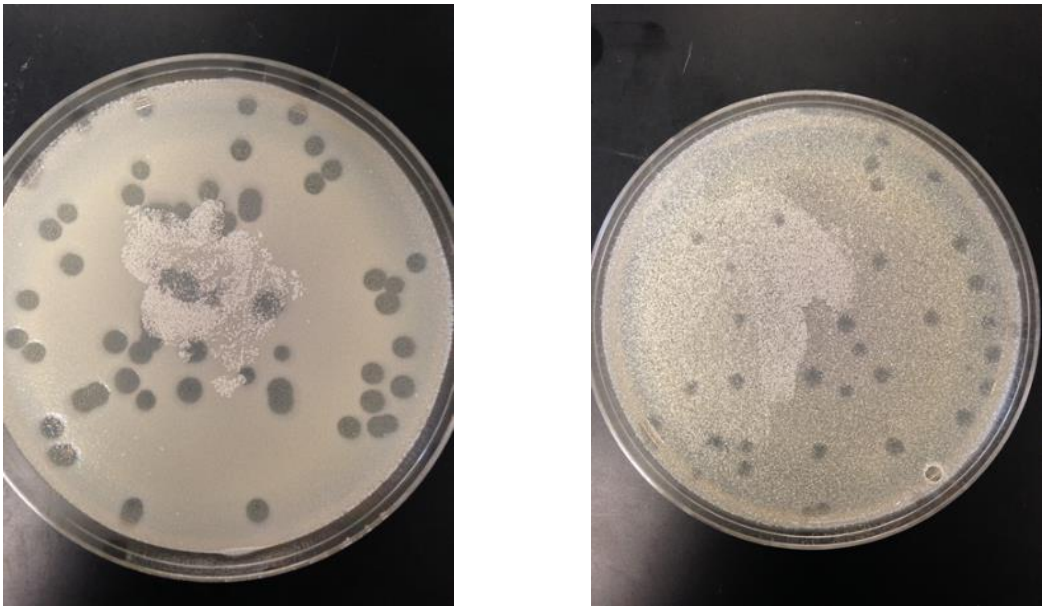

FIGURE 5: The picture of the plate on the left shows the bacteria and virus grown at 37°C with the virus at the 10^{-7} dilution. The picture of the plate on the right shows the bacteria and virus grown at 30°C with the virus at the 10^{-7} dilution. There is a difference in the plaque size between the two plates. The plaques on the plate on the left are much larger than the plaques of the plate on the right. This suggests that temperature plays a role in the infectivity rate of the isolated virus.

Future Research

Future research for studying the isolated virus includes taking an electron microscope picture of the virus to be able to visualize an individual virus instead of just seeing the clearing on the plate of bacteria of a group of viruses. Visualizing the virus will allow for there to be a better idea on exactly how the virus can infect the bacterial cell. The genome of the virus also needs to be sequenced to be able to annotate the genome. Annotating the genome will be essential in understanding the virus because knowing what genes the virus has will be helpful in determining how the virus can infect the bacterial cell. Also, knowing what genes the virus is made up will allow for comparison with other Mycobacterium virus genomes.

IMPLICATIONS

There are many positive impacts on healthcare that can come from this research. The first is having the opportunity to potentially find viruses that can infect bacteria that has become antibiotic resistant to multiple antibiotics. The goal of this research is to find viruses that will infect *Mycobacterium smegmatis*, but the overall purpose of the research for HHMI is to see if any of these viruses will infect *Mycobacterium tuberculosis* that has become antibiotic resistant (2). This is why this research is so imperative. It could eventually save many lives if a virus is found that can also infect and kill *Mycobacterium tuberculosis*. There are many places in the world where tuberculosis is still major problem in the healthcare field, and there are many people with cases of antibiotic resistant tuberculosis. If a virus can be found that can kill the bacteria, it will be able to help many people that would otherwise die from the disease.

There are already examples today of viruses being used to kill antibiotic resistant bacteria. One example is a new study that has been published about *Pseudomonas aeruginosa* with chronic lung infections (5). The study focuses on chronic lung infections such as cystic fibrosis. With this disease it is much more difficult for the body to fight off lung infections. The other problem is that many strains of *Pseudomonas aeruginosa* are becoming resistant to multiple antibiotics (5). This study has found a virus that can infect the bacteria and kill it. This a tremendous improvement for healthcare for individuals that are suffering from chronic lung infections. The virus is inhaled through a nasal spray so the virus can get to the patients lungs (5). The virus is able to kill the *Pseudomonas aeruginosa* bacteria which would otherwise potentially kill the person with the infection. This is known as phage therapy.

Phage therapy is now and will continue to be an important part of healthcare research because it is the future. There are many types of bacteria that are becoming antibiotic resistant to

multiple antibiotics. The world is coming to the point where alternatives to antibiotics need to be found, and phage therapy is one of the alternatives that has been proven to work.

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