Cantaurus, Vol. 27, 12-14, May 2019 © McPherson College Department of Natural Science

The Effects of Varying Temperatures on the Viral Infection Rates of *Escherichia coli* and *Bacillus subtilis*

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ABSTRACT

With the increase in the amount of medical cases involving antibiotic resistant bacteria, new treatments are needed to combat this new wave of diseases. The most viable option is quickly becoming bacteriophage therapy. Equipped with a series of natural processes, bacteriophages are naturally better equipped to combat bacterial infections than antibiotics. The purpose of this paper is to observe how temperature affects the effectiveness of the bacteriophages while infecting *Escherichia coli* and *Bacillus subtilis* using the Plaque Assay Method. The Plaque Assay Method involves using the number of plaque forming units (PFUs) and the concentration of the phage solution to determine the number of PFUs per milliliter of the phage stock solution. The research was not able to produce any conclusive results with a failure to produce plaques or bacteria cultures. Further literature research was able to explain possible reasons for this outcome. Further experimental research is needed to better determine to exact reason(s) and produce quantifiable results.

Keywords: *bacteriophage therapy, Escherichia coli, Bacillus subtilis, Plaque Assay Method, PFU,*

INTRODUCTION

The world is facing a new age of disease. Due to overuse, antibiotics have lost the ability to effectively cope with the bacteria that they are supposed to treat. Bacteria have evolved resistance to our current drugs, and pharmaceutical companies are struggling to produce new ones. Scientists have named these resistant bacteria “super bacteria.” They are becoming widespread across the globe. One estimate has stated that by 2050, 10 million people per year could die from these new super bacteria (Branswell, 2017).

Bacteriophage therapy have appeared as the optimal solution to combat these super bacteria. Several research groups have begun to study the use of bacteriophages as a treatment to bacterial infections, primarily using animals as test subjects. While still in the early stages, the research has shown some promise. A study done by Carrillo (et al. 2005) tested the colonization of broiler chickens by *Campylobacter jejuni*, a widespread and difficult to control infection. They then exposed the diseased chickens to bacteriophages CP8 and CP34. They found some success in combating the disease. Other studies are already looking at bacteriophages being used as a mean to control bacteria in fish farms (Almeida 2009).

Bacteriophages are ideal for combating these new super bacteria (El-Shibiny and El-Sahhan, 2017). The phages work by injecting a genome into the bacterial cell and integrating it into the genetic code. This causes the cell to begin producing more phages until the cell bursts, spreading the phages to the surrounding bacteria. While the bacteria are able to respond to the phages invasion with their own immune defenses, the phages are also able to respond. In a similar situation as with our own immune system, the phages are capable of evolving to work around the bacteria’s defenses. This makes the phages a natural solution.

However, there is more that needs to be understood before bacteriophage therapy is able to be viable as a way to control disease. The effects of environmental factors on the bacteriophages’ interactions with bacteria are largely unknown, with most research occurring on larger organisms (Dohm, Oguinn, and Turell, 2002). The human body requires certain bacteria in order to function properly. If these vital bacteria were to be killed off, then the consequences could be crippling or fatal. Without knowing the factors involved in the phages targeting mechanism, phage therapy is not viable.

My research is designed to begin to identify some potential factors that influence the infectivity of the bacteriophages. By identifying these key factors, scientists and doctors will be able to better understand bacteriophages in the hope of eliminating the super bacteria while leaving the vital bacteria untouched. This would allow the widespread use of bacteriophages to combat diseases in the human body without causing harm.

Temperature will be the primary factor to be observed. The human body maintains a temperature of around 37°C (98.6°F). Determining the role of temperature in the bacteriophages’ infectivity is crucial as altering the temperature of the human body too much could pose serious health risks to the person. Based on current research (Lowen, et al. 2007, Kilpatrick, et al. 2008) and knowledge, my hypothesis is that the temperature will impact the viral infection rates of bacteria.

MATERIALS AND METHODS

Escherichia coli and *Bacillus subtilis* were obtained from the Carolina Biological Supply along with the T4r coliphages, which had a stock concentration of $2x10^{10}$ plaque forming units per milliliter. The materials to make the agar were provided by the McPherson College Science Department.

Preparation began with nutrient broth. The broth consisted of 1.6g nutrient broth powder per 200mL of deionized water. Nine test tubes were fill with 9mL of the broth to be used to perform a 10-fold serial dilution of the phage stock. Two more test tubes were filled with 10mL of broth to be used to cultivate the parent cultures of *Escherichia coli* and *Bacillus subtilis.* All test tubes containing the nutrient broth were capped and autoclaved at 121ºC for approximately 45 minutes for sterilization. The test tubes were stored overnight in an incubator.

The next day, *Escherichia coli* and *Bacillus subtilis* were inoculated into the two test tubes containing 10mL of nutrient broth separately. The inoculated tubes were incubated for 24 hours at 27ºC to allow for culture growth. The hard agar, consisting of 28.75g nutrient agar mixed with 1.25 L deionized water, was prepared alongside the soft agar, 3.2g of nutrient broth powder and 3.6g of agar powder mixed with 400mL deionized water. Both agars were autoclaved in order to be sterilized. 60 plates of hard agar will be prepared using 20mL per plate to be stored for 24 hours in an inverted position in a refrigerator. The soft agar was distributed into 60 sterilized test tubes at a measurement of 8mL per tubes to be stored in an incubator for 24 hours at 45ºC to prevent gelling.

Plate inoculation took place in a sterilized fume hood beginning with the preparation of the phage 10-fold serial dilution. The T4r coliphage stock solution was diluted from the 10^{-1} to10^{-9} concentration range. The dilutions were thoroughly mixed to ensure equal distribution of the phages in solution. Two drops of the incubated bacteria and 0.1mL of phage dilution 10^{-5} were mixed using inversion with a tube of liquid soft agar before being poured over a plate of hard agar. The soft agar was allowed to gel to form a double-layered agar before the plate was covered. This process to occur for both *Escherichia coli* and *Bacillus subtilis* using the following phage dilutions: 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9}. 30 plates were used for each *Escherichia coli* and *Bacillus subtilis* to allow for three trials. 30 plates, half *Escherichia coli* and half *Bacillus subtilis,* were incubated at 27ºC and the remaining 30 plates were incubated at 37ºC.

Observation and recording of plates occurred using the Plaque Assay Method (Cappuccino, 2015). The number of plaque forming units, or PFUs, were recorded and the total number of PFUs per mL of the T4r coliphage stock solution were calculated. All agar plates and nutrient broth test tubes were collected, autoclaved in order to sterilize, and disposed of in the proper container.

RESULTS

The conclusion of the research experienced no quantifiable results. Four of the hard agar plates were disposed of due to early contamination of undesired bacterial growth before inoculation. The *Bacillus subtilis* soft agar tubes partially gelled in the test tubes after inoculation, resulting in a split in the sample. The *Escherichia coli* plates were able to be successfully incubated at 27ºC and 37ºC but showed no signs of plaque formation despite bacterial growth.

A second series of plates were prepared using increased phage concentrations and using only *Escherichia coli* due to being the only bacteria to exhibit growth. The phages concentration range was changed to 10^{-1} through 10^{-5} in the attempt to produce phage cultures. This series of *Escherichia coli* plates also failed to produce quantifiable results. The bacteria showed no signs of growth, having either failed to grow or having been completely eradicated by the phages.

DISCUSSION

The lack of plaque formations is due to a failure of the T4r coliphages to enter the bacterial cells and multiply. This failure could possibly be attributed to numerous other factors that were failed to be taken into consideration when preparing this experiment.

The T4r coliphages' accessibility to the bacteria may have influenced the apparent lack of plaque production. The use of solid media, such as the agar used, caused a loss of surface area that was available to the phages to absorb through, resulting in reducing the infection capabilities of the phages. Research accomplished by Guenther and his team in 2009 observed that the use of bacteriophages to combat bacteria was limited by their ability to diffuse in solid matrices, such as hot dogs.

The T4r coliphages were stored in a refrigerated environment for four months before being used. Research has shown that when the phages are incubated at refrigeration temperatures, the latent period is increased. This potentially increased the period of time from when the bacteria may have been infected by the phages and showing the symptoms of infection. If observed for a longer period of time, the cultures produced could possibly have eventually produced plaques (Ly-Chatain, 2014).

The method of delivery may also have been flawed. Phages are occasionally unable to diffuse through the bacterial membrane and may require a method of transport to reach the interior of the bacteria cells. Some researchers agree that the best delivery method is using non-pathogenic bacteria to bring the phages to the target cells (Inal JM. 2003).

Provided with a better understanding of how to resolve the complications that occurred during the

process of this research, it can be stated that changing method used in accordance with information previously discussed could produce quantifiable results. Bacteriophage therapy still remains the best alternative to combat antibiotic resistant bacteria. Their natural advantages and cost efficiency (Loc-Carillo and Abedon, 2011) allow the use of bacteriophage therapy to become a widespread practice while remaining effective.

ACKNOWLEDGEMENTS

I would like to thank Dr. Jonathan Frye and Dr. Wilgers, along with the rest of the Science Department members at McPherson College, for all their advice and patience with my research. I would also like to thank all the staff at McPherson College for their help and excellent teaching during my stay here. I would like to extend my thanks to all the researchers who provided the information needed to conduct my research. Lastly, I would like to thank my family for their unwavering support and love.

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