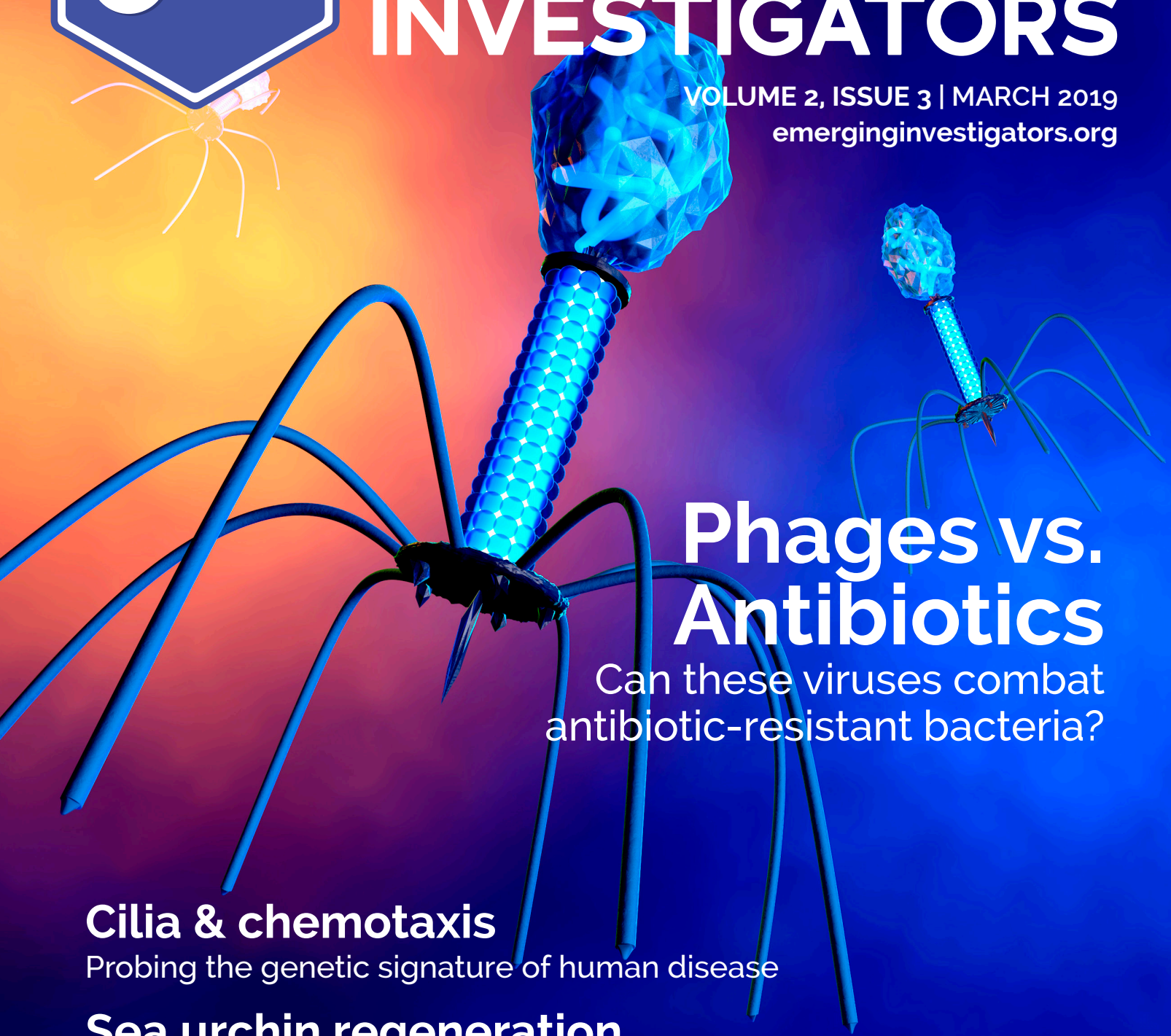
JOURNAL OF
EMERGING INVESTIGATORS

VOLUME 2, ISSUE 3 | MARCH 2019
emerginginvestigators.org



Phages vs. Antibiotics
Can these viruses combat antibiotic-resistant bacteria?

Cilia & chemotaxis
Probing the genetic signature of human disease

Sea urchin regeneration
The effects of fibroblast growth factor on spinal regrowth

Microplastic malady
Where do the tiny bits of plastic in waterways come from?

JOURNAL OF EMERGING INVESTIGATORS

The Journal of Emerging Investigators is an open-access journal that publishes original research in the biological and physical sciences that is written by middle and high school students. JEI provides students, under the guidance of a teacher or advisor, the opportunity to submit and gain feedback on original research and to publish their findings in a peer-reviewed scientific journal. Because grade-school students often lack access to formal research institutions, we expect that the work submitted by students may come from classroom-based projects, science fair projects, or other forms of mentor-supervised research.

JEI is a non-profit group run and operated by graduate students, postdoctoral fellows, and professors across the United States. JEI provides the opportunity for graduate students to contribute to the editorial and review process, and management of an academic journal.

Contents

VOLUME 2, ISSUE 3 | MARCH 2019

JOURNAL OF
EMERGING INVESTIGATORS

Article

Impact of *daf-25* and *daf-11* Mutations on Olfactory Function in *C. elegans*

Jason Gardner, Saman Bhamani, Kendyl Edwards, and Danielle Ereddia
Wheeler High School, Marietta, GA

SUMMARY

A plethora of life-threatening diseases found within humans are thought to result from malformed cilia. The genetic origins of such malformations can be analyzed by testing genes within *C. elegans* that are similar to human cilial genes. In *C. elegans*, chemotaxis and movement toward food are enabled by cilia. Wild-type *C. elegans* were expected to gravitate towards butanone, 2,3-pentanedione, and diacetyl, due to their association with to food, but were expected to move away from 2-nonanone. Chemotaxis assays were used to determine whether mutations in the cilia of two additional strains of nematodes (*daf-11* and *daf-25* mutant *C. elegans*) would alter the chemotaxis indices for these four odorants. Each of the three strains of *C. elegans* were placed on plates with sodium azide and one of the four odorants on either side. Both mutant strains were expected to display difficulty in making an association between the odorants and food due to their malformed cilia; however, we hypothesized that the *daf-25* mutants would be less effective at chemotaxis than the *daf-11* mutants. Our findings partially support this hypothesis. The wild-type *C. elegans* sensed and moved towards the odorants as expected more so than both of the mutant worms, and generally the *daf-11* mutant nematodes appeared to sense and move towards stimulants better than the *daf-25* mutants. There was no statistically significant difference between the senses of the two mutant strains while in the presence of this odorant.

INTRODUCTION

Many human disorders are caused by malformed cilia, such as Bardet-Biedl syndrome, retinopathies, obesity, situs inversus, and polycystic kidney disease (1). The genetic mechanisms behind Bardet-Biedl syndrome have not yet been discovered, but it is classified as a ciliary disorder. In this syndrome, vision loss is very common, along with obesity, delayed development of motor skills and speech, the presence of extra fingers/toes (phalanges), and lower amounts of sex hormones. Kidney abnormalities and loss of smell (anosmia) are also common in Bardet-Biedl syndrome (2). Retinopathy is caused when cilia involved in photoreceptors in the eyes malfunction, which results in vision impairment (3). Situs inversus is a condition where organs are reversed inside the human body. Situs inversus is seen in patients with Kartagener syndrome, which is also known as primary ciliary dyskinesia. Primary ciliary dyskinesia creates abnormal cilia on sperm cells, which can result in situs inversus (4). Cysts in polycystic kidney disease occur when cilia signaling is disrupted due to the malformation of cilia. The abnormal cilia are unable to sense the flow of fluid, which can impact gene expression and cause cysts to form (5).

Two human genes that help facilitate chemosensation have homologs in *C. elegans*. The human gene *NPR2* is homologous to the worm gene *daf-11*. The human gene *ANKMY2* is both homologous and orthologous to the worm gene *daf-25* (6). Mutations in genes that form cilia are responsible for the expression of a plethora of human disease phenotypes, as discussed above (1). These human diseases are greatly impacted by deformed cilia as a result of mutations in the human orthologs of *daf-11* and *daf-25* (2-5). It is unknown what percentage of the diseases mentioned previously are caused by mutations in these genes.

Genetic mutations of the genes in three distinct *C. elegans* neurons lead to the development of abnormal cilia and impaired senses. Similar defects in humans have the potential to cause the aforementioned diseases (1). The three neurons, AWA, AWC, and AWB, are directly related to the nematode's chemosensory abilities. These anterior neurons each correlate with either the repulsion or attraction of *C. elegans* to various odorants that may be present in their environment. AWA and AWC are both necessary for detecting when it is appropriate to move towards a given substance. AWB is necessary for detecting when it is appropriate to move away from a given substance (7). AWC detects the presence of odorants, such as benzaldehyde, butanone, isoamyl alcohol, 2,3-pentanedione, and 2,4,5-trimethylthiazole. AWA detects the presence of diacetyl, pyrazine, and 2,4,5-trimethylthiazole. Both sensory neurons contribute to *C. elegans'* movement towards their respective odorants (7). *C. elegans* are attracted to the aforementioned odorants because the odorants are produced by bacterial metabolism. Even though the worms do not eat the odorants, the smell of the odorants mimics their food because of the way the worms are raised. Therefore, the worms associate the odorants with food and see them as possible nourishment (7). AWB is responsible for their tendency to move away from some odorants, such as 2-nonanone. The reason behind this migration is still unknown (8).

Two genes that are highly relevant to the function of the AWA, AWC, and AWB neurons are *daf-25* and *daf-11*. The mutant strains of the *daf-25* and *daf-11* genes that are being utilized throughout the course of this experiment are *daf-25* (*m362*) and *daf-11* (*m47*). *daf-25* (*m362*) mutants are almost completely ineffective in chemotaxis in response to volatile odorants such as pyrazine, benzaldehyde, isoamyl alcohol, and trimethyl triazole (1). However, *daf-11* (*m47*) mutants are still moderately effective at chemotaxis in response to the volatile odorants listed above (1). Importantly, *daf-11* gene function is dependent on the presence of non-mutated *daf-*

25 (1).

The *daf-25* and *daf-11* genes result in the attraction or aversion of *C. elegans* towards certain odorants. In this study, we tested the odorants butanone, 2,3-pentanedione, 2-nonanone, and diacetyl. The AWC neuron controls the reaction to the odorants butanone and 2,3-pentanedione, while AWB controls the reaction to the odorant 2-nonanone and AWA controls the reaction to diacetyl. Because of the odorants' affiliation with the three neurons, it is known that the AWC and AWA odorants will be attractants, but that the AWB odorant will be a repellant (7). This new experiment differs from and extends upon a previous study by Jensen and colleagues which included the odorants benzaldehyde, trimethyl triazole, isoamyl alcohol, and pyrazine (1). The first three odorants are detected by the AWC neurons, while the last odorant, pyrazine, is detected by the AWA neuron. The results presented from our experiment have the potential to lend further credibility to the results presented in the previous study by Jensen *et al.*

In the previous study, information was gathered regarding the negative effects that mutant *daf-25* and *daf-11* genes have on the chemosensory capabilities of *C. elegans* while in the presence of pyrazine, benzaldehyde, isoamyl alcohol, and trimethyl triazole (1). This new experiment uses the same mutant genes as the experiment of Jensen *et al.*; however, we tested new odorants to increase the scope of the results. Because the two genes allow for *C. elegans* to have the chemosensory capabilities necessary for everyday life, the mutation of these two genes stifles such capabilities within the worms. Nevertheless, testing the mutant genes using a broader scope of odorants will allow for the hypothesis of this experiment to be tested in greater detail. In addition, further interrogation of the functional role *daf-11* and *daf-25* genes within *C. elegans* will prove helpful to learn more about the diseases and conditions that plague humans. Mutations in genes regulating cilia and their formation are responsible for a plethora of human disease phenotypes, such as the ones mentioned previously.

Our hypothesis was that wild-type worms would be attracted to butanone, 2,3-pentanedione, and diacetyl, resulting in a chemotaxis index close to 1. We also expected that the wild-type worms would be repelled from 2-nonanone, resulting in a chemotaxis index close to negative 1. The mutant worms would also move neither towards nor away from any of the four odorants, resulting in a chemotaxis index close to 0. We expected that the *daf-25* (*m362*) mutation would more greatly inhibit the chemotaxis capabilities of *C. elegans* than the *daf-11* (*m47*) mutation in an extensive number of volatile odorants. As previously stated, mutations of the *daf-25* gene impact the effectiveness and efficiency of both the *daf-25* and *daf-11* genes. Therefore, we expect a mutation in the *daf-25* gene to negatively influence the chemosensory, and therefore chemotaxis, capabilities of *C. elegans* to a greater extent than a mutation in the *daf-11* gene.

RESULTS

We conducted chemotaxis assays to determine the worms' tendencies to move toward or away from a set of given odorants. On petri dishes with Nematode Growth Medium, we pipetted an odorant on one side of the plate, close to the edge. We placed M9 buffer as a control on the opposite side. We drew a line down the middle of the plate, with each sector containing one odorant. We placed a sample of *C. elegans* in the middle of the plate, which represented sample size (Fig. 2). Worms were allowed to migrate to one side for thirty minutes. We counted the worms on each side and calculated a chemotaxis index. We averaged the indices across three trials to generate a final chemotaxis index for each condition (**Figure 1**).

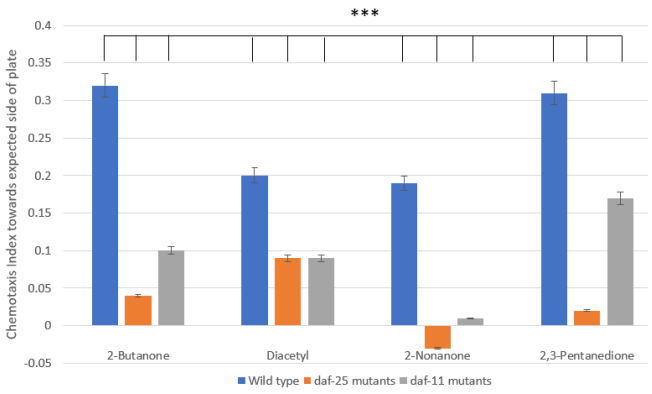


Figure 1. Average chemotaxis indices for each worm type, grouped by odorant. Chemotaxis indices represent how many worms traveled to each side of the plate. An index far from zero indicates a strong preference for that side of the plate, and an index close to zero indicates little preference for either side. n=3 for all tests. *** $p<0.001$ (two-way *ANOVA*).

The average chemotaxis indices are grouped by odorant and display the number of worms that went towards the expected side of the chemotaxis plates. The wild-type indices are higher than those of the *daf-25* and *daf-11* mutants, and the *daf-11* indices are slightly higher than those of the *daf-25* mutants (**Figure 1**). Wild-type worms were attracted to 2-butanone, 2,3-pentanedione, and diacetyl, while mutants were less attracted. Wild-type worms were repelled by 2-nonanone, but the odorant had little impact on the mutant worms. We then performed a two-way *ANOVA* test, a test of variance, on all chemotaxis data. We found that a worm's genotype and the odorant impact results of the chemotaxis assays ($p = 0.00096436$).

DISCUSSION

The goal of this study was to further the research done by Jensen *et al.* in order to add more significance to their conclusion. As seen in **Figure 1**, the hypothesis was mostly supported. A chemotaxis index close to zero indicates a minimal preference for one side or the other, while an index close to 1 or -1 indicates a strong preference by the group of worms. As hypothesized, the overall results displayed the wild-type nematodes as being significantly more interested than their mutated counterparts in moving towards butanone, 2,3-pentanedione, and diacetyl due to the association they are able to make between the odorants and sustenance. In general, the *daf-25* and *daf-11* mutants yielded indices that were significantly closer to zero than those of the wild-type strain when in the presence of butanone, 2,3-pentanedione, and diacetyl, indicating that they have disrupted cilia function.

Also, for reasons unknown, *C. elegans* are expected to be repelled from the odorant 2-nonanone. This phenomenon was supported by the wild-type strain data throughout this

experiment. The *daf-25* and *daf-11* mutant strains of *C. elegans* also verified claims about the difficulty they had detecting whether it is appropriate to move away from the odorant 2-nonanone, as their indices were -0.03 and 0.01, respectively (**Figure 1**). Our hypothesis, however, was only partially supported. While the differences between wild-type and mutants were as expected, the difference between mutant strains was not anticipated. According to results from previous studies, *C. elegans* that possess a mutated *daf-25* gene are practically ineffective at chemotaxis involving volatile odorants because of the effect a mutation in the *daf-25* gene has on both the *daf-25* and *daf-11* genes within a nematode (1). Results from tests on three out of the four odorants, butanone, 2,3-pentanedione, and 2-nonanone, aligned with previous studies, like those of Jensen *et al.* (1), because of the greater indices the *daf-11* mutants obtained. However, when testing one odorant (diacetyl), the *daf-11* mutant worms did not appear to have better chemotaxis abilities than the *daf-25* mutants, as they were awarded the same positive index: 0.09 (**Figure 1**). This suggests that the chemotaxis abilities of both strains are equal while in the presence of this odorant. It is unknown why this occurred and was most likely a result of a small number of trials. Also shown in **Figure 1**, the *daf-11* mutants obtained a greater index while in the presence of 2-nonanone than the *daf-25* mutants. This suggests that the *daf-11* mutant worms are more adept at identifying when it is appropriate to move away from the 2-nonanone odorant than the *daf-25* mutants. Therefore, the results acquired throughout this experiment partially support the hypothesis that initiated our experiment, as well as the conclusions of various other studies (1).

If this experiment were to be repeated, a few details would be altered. More chemotaxis assays would be completed to increase the statistical power of our results. More trials could increase the validity of the claims we are able to make from our data. Although there are questions of validity concerning the accuracy of manually counting the number of worms on each side of the plate, assuming the counting of worms was accurate, the two-variable *ANOVA* validates our data and deems it statistically significant. However, additional trials could verify our hypothesis further and confirm our results. Another element that could be improved in later experiments would be the aseptic technique throughout this experiment, such as sterilizing workspaces more often and with greater care. Taking greater precaution when performing this experiment could result in more accurate data. The last improvement for future experiments would be to utilize kimwipes to wick away excess M9 buffer. Some worms were rendered immobile due to excess M9 buffer, which could have altered their chemotactic ability and therefore the data.

This experiment's data informs many future studies. Existing protocols may be expanded upon in the future by testing the result of new volatile odorants, such as benzaldehyde, isoamyl alcohol, and 2,4,5-trimethylthiazole. One could also conduct this experiment with a dual mutant strain of *daf-25* and *daf-11*, as this may have an impact on a strain's response to different odorants. Lastly, testing a *daf-19* strain would also be helpful, as this strain regulates many cilia-related genes and is critical in the development of sensory cilia (1). Discovering more about the role mutations play in the chemosensory abilities of *C. elegans* will allow humans to gain a greater understanding of how mutations of chemosensory-regulating genes may impact their lives.

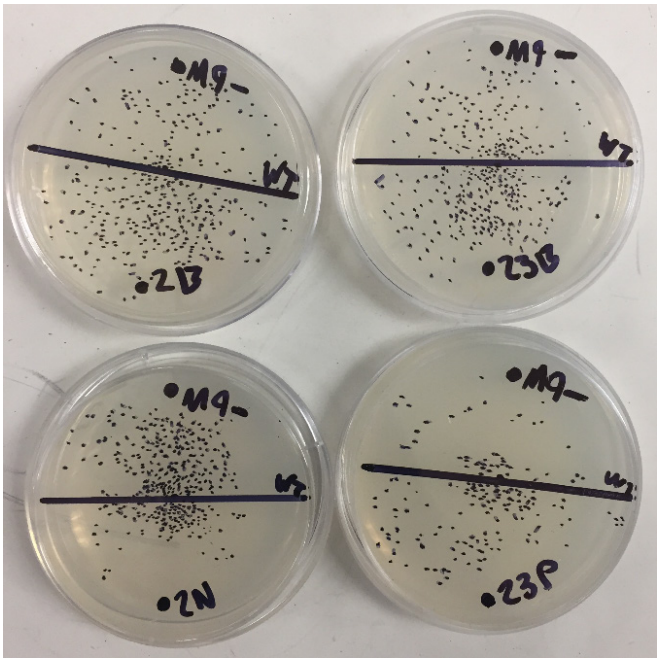


Figure 2. Chemotaxis plates for trial 1 of wild-type worms. An image of the four chemotaxis plates from trial 1 of the wild-type chemotaxis assays. It shows that the worms were attracted to butanone, 2,3-pentanedione, and diacetyl, but repelled by 2-nonanone.

METHODS

Nematode Growth Media Plate Preparation

A 1-liter Erlenmeyer flask was used to mix the solution for the NGM agar, 3 g NaCl, 17 g agar, 2.5 g peptone, and 975 mL H_2O. It was then autoclaved for 50 minutes and cooled in a 55°C water bath for 15 minutes. After it was cooled, 1 mL 1M $CaCl_2$, 1 mL 5 mg/mL cholesterol, 1 mL 1 M $MgSO_4$, and 25 mL 1 M KPO_4 buffer were added. It was then mixed by swirling and subsequently poured into 6-cm petri dishes until they were ⅔ full (9).

Bacterial Preparation

A single colony of *E. coli* OP50 was selected from a plate and grown in LB overnight at 37°C with agitation. An L-rod was then used to transfer OP50 to the center of 15 NGM plates. They were incubated at room temperature for 48 hours, and then stored upside down at 4°C or were used immediately (10).

Worm Growth

A worm sample was added to a pre-prepared NGM plate containing *E. coli*. This plate was then left alone for 4–7 days. M9 Buffer was used to transfer worms to a new plate that already contained *E. coli*. The plate was incubated at room temperature for two days. This was done to give the worms a constant food source since their given mutations prevent dauer formation.

Washing Worm Samples

A pipette was used to transfer 1 mL M9 buffer on the plate containing the subculture of *C. elegans*. The plate was tilted until the whole surface was covered and was left to sit for 30

seconds. Afterwards, the plate was tilted at a 45° angle to allow *C. elegans* to pool at the edge of the plate. A pipette was then used to transfer this liquid into a microcentrifuge tube. After the *C. elegans* settled at the bottom, the supernatant was removed from the top, and 1 ml M9 buffer was added back into the microcentrifuge tube. This wash step was repeated two more times, but on the last time, approximately 200 µL M9 buffer was left in the tube above the *C. elegans* (11).

Chemotaxis Assay

First, a pre-made NGM plate was labeled and divided into a control side and a chemical side. Then, 2 µL 0.5 M sodium azide was added to the outermost spots on opposite sides of the plate. This immobilized the worms once they reached the far sides of the plate. About six hours later, 2 µL of the designated odorant was added to the test side of the plate, and 10 µL of M9 buffer was added to the control side. A micropipette was used to transfer 4 µL of the *C. elegans* sample from the microcentrifuge tube with the same label. The excess M9 buffer was absorbed using a tissue so as to not hinder the worms' movement. After 10 minutes passed and it was ensured that the *C. elegans* were traveling across the agar, they were left at room temperature for 30 minutes face up. The plates were held against a bright light and worms were counted on their respective sides, and a chemotaxis index was calculated (**Figure 2**). Each chemotaxis index was calculated by subtracting the number of *C. elegans* on the control side from the number of *C. elegans* on the odorant side. This number was then divided by the total number of worms on that plate. A chemotaxis index was calculated for every plate that contained one of the three strains of *C. elegans*, as well as one of the four odorants used during this experiment. "Worm Growth," "Washing Worm Samples," and "Chemotaxis Assay" were repeated for each odorant and *C. elegans* strain (11).

Data Analysis

A two-variable *ANOVA* was utilized to determine the significance of the results acquired throughout the experiment. A two-variable *ANOVA* recognizes the influence two independent variables may have on the dependent variable of an experiment. For this particular experiment, the independent variables are the three strains of worms (wild-type *C. elegans*, *daf-11* mutant *C. elegans*, and *daf-25* mutant *C. elegans*) and the four odorants (butanone, 2,3-pentanedione, diacetyl, and 2-nonanone), while the dependent variable is the chemotaxis index. The indices are based on the chemotaxis abilities each strain of *C. elegans* has in the presence of four odorants.

Received: January 04, 2018
Accepted: November 17, 2018
Published: February 2 , 2019

REFERENCES

1. Jensen, Victor L., *et al.* "Localization of a Guanylyl Cyclase to Chemosensory Cilia Requires the Novel Ciliary MYND Domain Protein DAF-25." *PLoS Genetics*, vol. 6, no. 11, 2010, doi:10.1371/journal.pgen.1001199.
2. "Bardet-Biedl Syndrome - Genetics Home Reference - NIH." U.S. National Library of Medicine, *National Institutes of Health*, Accessed: 15 May 2018, ghr.nlm.nih.gov/condition/bardet-biedl-syndrome#definition.
3. Ghosh, Amiya K., *et al.* "Human Retinopathy-Associated Ciliary Protein Retinitis Pigmentosa GTPase Regulator Mediates Cilia-Dependent Vertebrate Development." *Human Molecular Genetics*, vol. 19, no. 1, 2009, pp. 90–98., doi:10.1093/hmg/ddp469.
4. Guichard, Cécile, *et al.* "Axonemal Dynein Intermediate-Chain Gene (DNAI1) Mutations Result in Situs Inversus and Primary Ciliary Dyskinesia (Kartagener Syndrome)." *The American Journal of Human Genetics*, vol. 68, no. 4, 23 Feb. 2001, pp. 1030–1035., doi:10.1086/319511.
5. Yoder, B. K. "Role of Primary Cilia in the Pathogenesis of Polycystic Kidney Disease." *Journal of the American Society of Nephrology*, vol. 18, no. 5, 2007, pp. 1381–1388., doi:10.1681/asn.2006111215.
6. Zerbino, Daniel R, *et al.* "Ensembl 2018." *Nucleic Acids Research*, vol. 46, no. D1, 16 Nov. 2017, doi:10.1093/nar/gkx1098.
7. Bargmann, Cornelia. "Chemosensation in C. Elegans." *WormBook*, 25 Oct. 2006, doi:10.1895/wormbook.1.123.1.
8. Troemel, Emily R, *et al.* "Reprogramming Chemotaxis Responses: Sensory Neurons Define Olfactory Preferences in C. Elegans." *Cell*, vol. 91, no. 2, 17 Oct. 1997, pp. 161–169., doi:10.1016/s0092-8674(00)80399-2.
9. Stiernagle, Theresa. "Maintenance of C. Elegans." *WormBook*, 11 Feb. 2006, doi:10.1895/wormbook.1.101.1.
10. Porta-De-La-Riva, Montserrat, *et al.* "Basic Caenorhabditis Elegans Methods: Synchronization and Observation." *Journal of Visualized Experiments*, no. 64, 10 June 2012, doi:10.3791/4019.
11. Hart, Anne C. "Behavior." *WormBook*, 3 July 2006, doi:10.1895/wormbook.1.87.1.

JOURNAL OF
EMERGING INVESTIGATORS

Article

Determining the Effects of Fibroblast Growth Factor 2 on the Regenerative Abilities of *Echinometra lucunter* Sea Urchins

Danielle Kisling, Maya Lee, Ritika Sadalge, Avni Sawant, Danielle Ereddia
Wheeler High School, Marietta, Georgia

SUMMARY

Advances in regeneration have the potential to benefit the healthcare field through contributions to wound healing, organ transplants, and many more related technologies. This experiment was performed to help contribute to further research in vertebral regeneration, as humans' capacity to regenerate is mostly limited to slower and less complex forms of regrowth. Due to their exceptional ability to regenerate entire bodily appendages, we used sea urchins of the species *Echinometra lucenter* as models for the study of regeneration. This experiment was constructed to examine the effects of fibroblast growth factor 2 (FGF2) on spinal regeneration time in the urchins. We hypothesized that the addition of this growth factor would cause urchins to regenerate a larger amount of their spinal tissue 14 days after severance. Although the mean percent regeneration of the experimental group was higher than that of the control, the results were not statistically significant, which reflects a possible lack of correlation between FGF2 and an increased regenerative ability. Further testing is required to discover the possible implications of the data and effect of FGF2 on humans.

INTRODUCTION

Almost all organisms possess a regenerative capacity to some extent, but for humans and most other mammals, this capacity is largely limited to smaller-scale regeneration processes such as wound healing and repair (1). Within humans, the exception to this is the liver, which has the ability to repair lost mass and grow to fit the size of the organism it inhabits (2). Each day, 20 people in the United States die while waiting for an organ transplant (3). Thus, the ability to stimulate regeneration in human organs other than the liver would have a profound impact on the scientific and medical community. It would reduce a recipient's need to rely on an organ donation, as organs could potentially be grown in laboratories (4).

Echinometra lucunter, commonly known as the rock boring sea urchin, has a unique ability to regenerate spines and tube feet and can potentially provide a model for regenerative growth. Echinoderms are ideal organisms due to their relatively quick ability to fully regenerate external appendages, most likely due to their abundance of multipotent cells. Furthermore, sea urchins are non-chordate deuterostomes and are related phylogenetically to humans, so they can also provide insight into mechanisms of regeneration in vertebrates (5). By examining a process that contributes to a greater efficiency of regeneration in echinoderms, we can identify potential factors that regulate regeneration in vertebrates (6).

A previous study's results indicated that the mechanism related to spine and tube feet regeneration in adult sea urchins required a functional Notch signaling pathway, which interacts with other signaling pathways to stimulate growth. This finding supported the hypothesis that the sea urchins that were given the mitotic inhibitor were unable to regrow their amputated appendages (5). Another study examined the effects of varying environmental conditions, specifically ocean acidification, upon adult sea urchins' ability to re-form body structures. The study discovered that the increase of atmospheric carbon dioxide greatly affected the seawater's chemistry and though the spines were able to regenerate, their structural integrity was greatly compromised (7).

Heparan sulfate proteoglycans (HSPGs) are glycoprotein components of the extracellular matrix of all animal cells (8). It is a suitable glycoprotein to focus on in our study of regeneration in sea urchins because it plays a large role in regulatory processes such as wound repair, coagulation, and cell migration (9). In addition, HSPGs interact with a variety of membrane receptors to promote extracellular matrix attachment and a variety of other extracellular interactions (9). For example, the liver has a high regenerative ability due to membrane HSPGs operating as endocytic receptors for the passage of ligands. In fact, studies have shown that HSPGs may assist in recovery from acute liver injury (9). Also, HSPGs can assist the process of growth factor dispersal (10).

In addition, HSPGs are critical in stem cell maintenance. In fact, when stem cells lack HSPGs due to an *Ext1* gene deficiency, they often lose their ability to differentiate and respond to growth factors (11). This highlights the vitality of HSPGs when it comes to the regeneration and differentiation of the cells that create animal tissue. Some of the growth factors capable of binding to HSPGs are fibroblast growth factors.

Fibroblast growth factors function to control the growth and differentiation of progenitor cells during embryonic development and organogenesis. They bind to heparan sulfate proteoglycans and through the use of signaling pathways, they regulate metabolic processes in mature tissues such as tissue repair and regeneration (12). Studies show that fibroblast growth factors (FGFs) likely play an important role in the successful regeneration of liver tissue because their inhibition diminished liver regeneration in rodents (12). In one study, mice that lacked *Fgf15* exhibited defects in regeneration due to an inability to properly regulate the cell cycle. Because researchers have found that echinoderms are capable of binding FGF2, we determined it to be the ideal growth factor for a study involving these animals (13). FGF2 increases the production of cells which stimulates healing. After two days of low dose FGF2 present in the skeletal system of the mice, the rate of cell growth increased by 10% (14).

FGF2 helps to promote angiogenesis, which can help

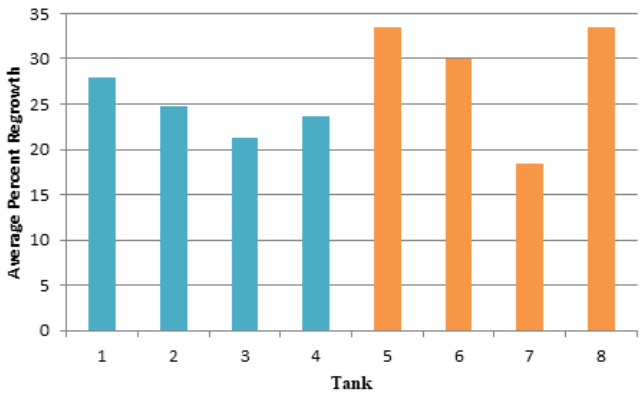


Figure 1. Average percent urchin regeneration by tank. Tanks 1, 4, 7, and 8 were each 10 gallons and contained 3–4 urchins per tank. Tanks 2, 3, 5, and 6 were 5-gallon tanks and contained 2–3 urchins per tank. Spine length after amputation was compared to spine length prior to amputation. Each bar represents the average percent regeneration per tank. Blue bars represent the control group of urchins and the orange bars represent the group of urchins that received FGF2.

individuals recover better and retain more blood flow (15). Discovering a method to promote regeneration in a variety of human tissues and blood vessels would undoubtedly have a monumental impact on modern science and medicine. This study examined FGF2's effect on regeneration in the *E. lucunter* species. By studying the rate at which the urchins' appendages regenerate, the results can potentially support the possibility of growth factors increasing regenerative abilities in more organisms.

We hypothesized that the addition of FGF2 solution into the system of the *E. lucunter* would make the time of regeneration approximately 10% faster, as seen in previous studies (14). Therefore, we predicted that a single urchin spine with FGF2 would regenerate more of its original length in 14 days than the urchins of the control group (5). After experimentation, the mean percent regeneration of the experimental group was 28.86%, which was higher than that of the control which was 24.44%. The calculated *p*-value was 0.09.

RESULTS

Tanks 1-4 held the control group of urchins while tanks 5-8 held the experimental group of urchins. Discrepancies between the number of urchins at the start and end of the experiment signify that deaths occurred (**Table 1**). Two deaths occurred in Tank 1, one death occurred in Tanks 4, 6, 7, and 8, and no deaths occurred in Tanks 2, 3, and 5.

The control group (blue) had average spine regrowths of 27.99% (Tank 1), 24.79% (Tank 2), 21.35% (Tank 3), and 23.63% (Tank 4) (**Figure 1**). The experimental group (orange) has averages of 33.48% (Tank 5), 29.96% (Tank 6), 18.49% (Tank 7), and 33.49% (Tank 8). These percentages were found by cutting spines before and after FGF2 administration and comparing lengths.

The control group had a lower average percent of spine regrowth (24.44%) but also had a lower standard error (1.38), whereas the experimental group had a higher average percent of spine regrowth (28.86%) with a larger standard error (3.55) (**Figure 2**). The error range of the experimental group

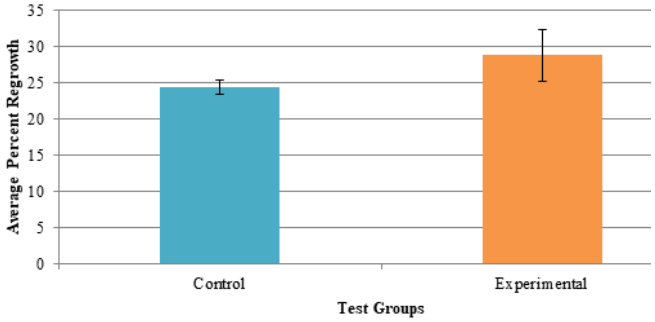


Figure 2. Average percent regrowth for both the control and experimental groups. The control group contained tanks 1–4 and held 11 urchins. The experimental group contained tanks 5–8 and held 13 urchins. The blue bar represents the average spine regeneration percentage of all the tanks in the control group and the orange bar represents the average spine regeneration percentage of all the tanks in the experimental group. The error bars indicate standard error. p=0.09 (one-tailed t-test).

is 23.06-25.82 and the standard error of the control group is 25.31-32.41.

A test of normality was also conducted and the values were plotted. The plot was roughly linear, thus indicating that the data was approximately normally distributed. Once this was established, a one-tailed *t*-test was conducted. The *p*-value was 0.090521. A *p*-value of 0.09 indicates that there is a 9% chance that these results would occur if FGF2 had no effect on regeneration. This means that our hypothesis was not definitively supported as a 5% value would be required to suggest statistical significance.

DISCUSSION

The sea urchins in our study showed a greater average percent regrowth for the experimental group compared to the control group (**Figure 2**). However, since the standard error for the data is so large, the FGF2 may not have had an equal effect in all experimental tanks. In particular, Tank 7 had a significantly lower percent regrowth than the rest of the experimental group (**Figure 1**). By the end of the regeneration period, the 10 urchins in the experimental tanks regenerated on

Tank	Vol. (gal)	Filter type	Group	No. of urchins at start	No. of urchins at end
1	10	Tetra Whisper	Control	4	2
2	5	Tetra Whisper	Control	3	3
3	5	Tetra Whisper	Control	2	2
4	10	Tetra Whisper	Control	2	1
5	5	Penn-Plrx Cascade Heat (Model CH850)	Exper.	3	3
6	5	Tetra Whisper	Exper.	3	2
7	10	Tetra Whsiper	Exper.	4	3
8	10	Tetra Whisper	Exper.	3	2

Table 1. Tank parameters and number of urchins in each tank at the start and conclusion of the experiment.

average about 18% more spinal tissue than the 8 in the control group. This indicates that the FGF2 may have acted as an injury response agent and induced faster spinal regeneration. However, the *t*-test showed a *p*-value of 0.09, indicating that the data was not statistically significant.

One study showed an average spine regeneration of around 44% in 14 days without any growth factors added (5). Other researchers found that the addition of FGF2 causes spines to regenerate 48.4% of their original length in 14 days (14). The current study found that spines regenerated an average of 29% of their original length in 14 days.

Hence, our results showed a deficiency in the average percent regrowth compared to what was predicted. The reason for this may have been due to high stress levels in the sea urchins, indicated in the experiment when many spines fell off (16). This would have a significant impact on the data as there was no way to measure the lengths of prematurely detached spines. The stress could have been caused by inadequate filtration, mold in the bottom of the tanks from the live rock, and overhandling of the urchins. Because the water conditions varied between tanks, each tank was looked at separately. The varying conditions like mold and filter type likely impacted stress levels and therefore regeneration. Mold was present in Tank 7, which ended up having the lowest regrowth rate. Also, the results were compromised because some of the sea urchins died mid-experiment. The sea urchins that died were in tanks 1, 4, 6, 7, and 8. This could have been due to the aforementioned varying tank conditions. Therefore, there was a smaller sample size than what would have been optimal for this experiment, and this undoubtedly could have made it more difficult to draw conclusions because the statistical analysis is not as powerful.

In future experiments, steps could be taken to reduce variability. For example, a thinner pair of dissection scissors could be used to cut with a higher degree of precision. It was often difficult to cut close to the test (the skeleton of the urchin) using a large pair of scissors because of the surrounding spines. Additionally, a higher-quality waterproof adhesive would be advisable for tagging the urchins as the tape used in this experiment would occasionally detach from the specimen. There were also discrepancies between the filters in the different aquariums as some were different brands and therefore higher quality than others. This may have affected the health and stress levels of some of the urchins, as a low-quality filter possibly could have caused stress and contributed to their deaths. Accordingly, it is recommended that identical filters be utilized in each aquatic habitat in future experiments as it will reduce the likelihood of an additional variable having an impact on the results. Another possible factor that was linked to the variance in data was the amount of FGF2 administered per urchin. The amount of FGF2 given can be based on urchin size, so ideally all urchins would have been the same or similar sizes, allowing the administration of a standard dose of FGF2.

To further extend this experiment, rather than focusing solely on regeneration of appendages, morphallaxis in the *Hydra vulgaris* could be studied in order to gain an understanding of the effect of FGF2 on the process of full-body morphallactic regeneration after bisection. Additionally, the effect of FGF2 on tail regeneration in amphibians could be tested to examine if its effects transfer to more complex organisms that contain more highly differentiated tissues than those of *E. lucunter*. In the future, these studies could potentially be used to enhance understanding of regeneration in humans and could possibly be applied to methods of promoting faster rates of regeneration in human liver.

METHODS

Saltwater Tank and Habitat Set-up

Four ten-gallon tanks and four five-gallon tanks were set up for the sea urchins' habitat. Tanks 1, 4, 7, and 8 were the 10-gallon tanks and tanks 2, 3, 5, and 6 were the 5-gallon tanks. They were placed in conditions with 35 parts per thousand of salt, which is the salt content of their natural habitat, the Atlantic Ocean. Two pounds of live rock were kept in the ten-gallon tanks and one pound was kept in the five-gallon tanks to provide nutrition and shelter for the urchins. The temperature of the tank was maintained between 23.9–27.8 ˚C with constant filtration. The *E. lucunter* were fed eight cubic millimeters of algal-agar cube weekly. The algal-agar cubes were made by mixing 3.6 g of agar with 100 mL water and heating until boiling. Then 2 grams of green algae (crushed by mortar and pestle into fine powder) were added to 16 mL of water and were stirred to ensure uniform mixing. Next, the algae solution and the agar solution were mixed together. Then the algal-agar mixture was spread onto a microscope slide covered with wax paper, left to solidify, and cut into cubes of 2 mm^3.

FGF2 Administration

0.01 g (10 µL) of FGF2 obtained from Prospec Protein Specialist were mixed with 5,000 µl of distilled water and were then fed to the 13 experimental *E. lucunter* by inserting an insulin needle into their oral cavity to ensure that each urchin received the complete 501 µL of solution needed, which in turn gives them the full 5 ng of FGF2. The 13 experimental and 11 control urchins were then left for 2 days so that 10 % of FGF2 was released and was able to bind to the heparan sulfate located at the surface of the cells (14).

Identification of Urchins

If a tank contained two urchins of similar size, then one urchin was labeled using a strip of waterproof tape around a single spine. Tanks 1, 2, 5, and 8 all contained two urchins that were similarly sized, so one of each of those urchins was tagged to differentiate it from the other.

Spine Severance

Two days after the *E. lucunter* experimental group was fed the FGF2, four spines were cut off from one ambulacrum section of each sea urchin from both groups using dissection scissors. For future identification purposes, spines from each urchin were cut in a vertical line and numbered one to four (from top to bottom). The lengths of the severed appendages were measured using a caliper and recorded. After being amputated, sea urchins were left to recover overnight without disruption for 24 hours. The regrowth of the spines was then monitored over a course of 14 days (5).

Measuring Regeneration of the Spines

The lengths of the four regenerated spines of each urchin were measured at the end of the full two-week period. On the final day of the experiment, the sea urchins were removed from the tank, placed in a shallow container, and allowed to

relax. Then, the regenerated spines were cut off with the dissection scissors. The electronic caliper was placed adjacent to the newly cut off spine, and the newly cut-off spine was then measured and compared to the length of the previously amputated spine and the percent regeneration was calculated. This growth showed how fast the regeneration of the spines occurred, which allowed the amount of regeneration of the experimental group to be compared to that of the control group (5). The average growth of each tank was taken and the average growth of the experimental group and the control group as a whole was calculated to determine whether the hypothesis was supported. A *t*-test was performed in Excel Spreadsheets to determine if the data was significant.

Received: June 04, 2018
Accepted: September 30, 2018
Published: February 12, 2019

REFERENCES

1. Goss, Richard Johnson. "Regeneration." Encyclopædia Britannica, *Encyclopædia Britannica, Inc.*, 22 Aug. 2016
2. Michalopoulos, George K. "Liver Regeneration." *Journal of Cellular Physiology*, vol. 213, no. 2, Nov. 2007, pp. 286–300., doi:10.1002/jcp.21172.
3. "Data." *United Network for Organ Sharing*, 28 June 2018
4. "Factsheet." *National Institutes of Health*, U.S. Department of Health and Human Services
5. Reinardy, H. C., Emerson, C. E., Manley, J. M., & Bodnar, A. G. "Tissue Regeneration and Biomineralization in Sea Urchins: Role of Notch Signaling and Presence of Stem Cell Markers." *PloS One*. vol. 10, no. 8. 12 Aug. 2015.
6. Vickery, M C, *et al.* "Regeneration in Echinoderm Larvae." *Microscopy Research and Technique*. vol. 55, no. 6, 21 Dec. 2001, pp. 464-473.
7. Emerson, C. E., Reinardy, H. C., Bates, N. R., & Bodnar, A. G. "Ocean acidification impacts spine integrity but not regenerative capacity of spines and tube feet in adult sea urchins." *Royal Society Open Science*. vol. 4, no. 5, 17 May 2017.
8. Shriver, Z., Capila, I., Venkataraman, G., & Sasisekharan, R. "Heparin and Heparan Sulfate: Analyzing Structure and Microheterogeneity." *Handbook of Experimental Pharmacology*. No. 207, 2012.
9. Apte, U., & Kaplowitz, N. "Heparan sulfate promotes recovery from acute liver injury: Inhibition of progressive cell death or enhanced regeneration." *Hepatology*. Vol. 66, no. 5, November 2017.
10. Matsuo, I., & Kimura-Yoshida, C. "Extracellular distribution of diffusible growth factors controlled by heparan sulfate proteoglycans during mammalian embryogenesis." *Philosophical Transactions of the Royal Society of London*. Vol. 369, no. 1657, 05 Dec. 2014.
11. Sarrazin, S., Lamanna, W. C., & Esko, J. D. "Heparan Sulfate Proteoglycans." *Cold Spring Harbor perspectives in biology*. vol. 3, no. 7, July 2011.
12. Böhm, F., Köhler, U. A., Speicher, T., & Werner, S. "Regulation of liver regeneration by growth factors and cytokines." *EMBO molecular medicine*. vol. 2, no. 8, Aug. 2010.
13. Ramachandra, R., Namburi, R., Ortega-Martinez, O., Shi, X., Zaia, J., Dupont, S. T., *et al.* "Brittlestars contain highly sulfated chondroitin sulfates/dermatan sulfates that promote fibroblast growth factor 2-induced cell signaling." *Glycobiology*. vol. 24, no. 2, 13 Nov. 2013.
14. Charles, L. F., Woodman, J. L., Ueno, D., Gronowicz, G., Hurley, M. M., & Kuhn, L. T. "Effects of low dose FGF2 and BMP-2 on healing of calvarial defects in old mice." *Experimental gerontology*. vol. 64, Apr. 2015.
15. Tu, Min, *et al.* "Vasohibin 2 promotes human luminal breast cancer angiogenesis in a non-paracrine manner via transcriptional activation of fibroblast growth factor 2." *Cancer Letters*. vol. 383, no. 2, Dec. 2016.
16. "Adult Sea Urchins." Sea Urchin Embryology. *Stanford University*. (n.d.).

JOURNAL OF
EMERGING INVESTIGATORS

Article

A quantitative analysis of the proliferation of microplastics in Williamston's waterways

Luke Schafer, Hudson Yu, and Joe Rasmus
Williamston High School, Williamston, MI

SUMMARY

One of the most concerning aspects of human progress is the spread of pollution. Microplastic pollution is only a small part of this issue, but a relevant one nevertheless. Plastic debris can disrupt marine ecosystems, spread contaminants, and take years to naturally degrade. Our aim for this study was to establish an understanding of the scope of Williamston, Michigan's microplastics problem, as well as to attempt to find the source of these plastics. We sampled from four sites from the Red Cedar River in the Williamston School District. Sites were chosen due to their proximity in relation to the boundary of the school district, with samples collected both up and downstream of the wastewater treatment plant. In analyzing our samples, we used an aspirator vacuum to filter the water we collected, left the filters in an incubator to dry for 48 hours, and then counted microplastics under a microscope by systematically scanning through gridded filter paper. We found a general trend of increasing concentrations of microplastics from upstream to downstream, but we were not able to locate the source of Williamston's microplastics pollution. Originally, we hypothesized that the Williamston Wastewater Treatment Plant was the primary contributor to Williamston's microplastics pollution, but we could not find statistically sufficient evidence to confirm this theory. Further research is needed to determine whether the Wastewater Treatment Plant or another source is responsible for the microplastics pollution.

INTRODUCTION

Currently, 8 million tons of plastic waste enters the marine ecosystem each year, much of which is in the form of tiny microplastics, which are plastic particles smaller than 5mm (1). If these plastics are allowed to build up at their current rate, without any reduction, an ecological disaster may be inevitable. Even now we may have already reached a tipping point. The United Nations estimates that there are 51 trillion microplastics in the ocean (2). Microplastics are incredibly harmful to the environment (3). When found in high concentrations, they can spread contaminants, endanger wildlife, and damage marine ecosystems. In April 2018, a dead sperm whale discovered by Spanish researchers was found to contain 64 pounds of plastics in its digestive system (2). Plastic is a substance that is made to last, and as a result, microplastic particles can take decades to degrade, allowing for their buildup. This fact further emphasizes the necessity for action, as the global microplastic threat is frequently overlooked (4). A major reason for this could be the "out of sight, out of mind" mentality that many people subscribe to, seeing as most people do not come face-to-face with extensive buildup of microplastics on a daily basis.

The purpose of this study was to gain an understanding of Williamston's microplastics accumulation and the extent of the problem in the Red Cedar River. We focused on two aspects of this issue: the origin of Williamston's microplastics and their concentration. If we have detailed knowledge of the concentration of microplastics, it will allow us to gauge the scope of Williamston's microplastics problem and more importantly its potential impact. In addition, knowing the origin of microplastics will aid in containing the issue at hand.

In this situation, there were clear independent and dependent variables. The independent variable was the location that our team decided to collect our water samples. These sample locations were located near notable sites on the Red Cedar that fall within the Williamston School District's boundaries (**Figure 1**). The dependent variable, or the focus of our observations, was the concentration of microplastics in our samples. We assumed that the number of microplastics per liter that we found would be dependent upon where we sampled.

The results of our study were difficult to predict, but based on other studies, we expected relatively low microplastic levels. For example, Baldwin et al. (2016) found as little as 0.002 particles per liter in rivers in the Great Lakes Basin (5). However, Castañeda et al. (2014) found over 87 particles per liter (6). Based on this disparity between the studies, it was hard to predict the amount of microplastics that we would find. However, our location is relatively rural and therefore similar to the area analyzed by the first study, which led us to hypothesize that Williamston would have a low concentration of microplastics, somewhere in the range of 0-10 particles per liter. We also hypothesized that the Williamston Wastewater Treatment Plant would be the primary contributor of microplastics into the river. This hypothesis was derived from research that occurred at the University of Leeds in the United Kingdom that linked wastewater treatment plants to microplastics pollution (7).

According to the US Geological Survey, microplastics

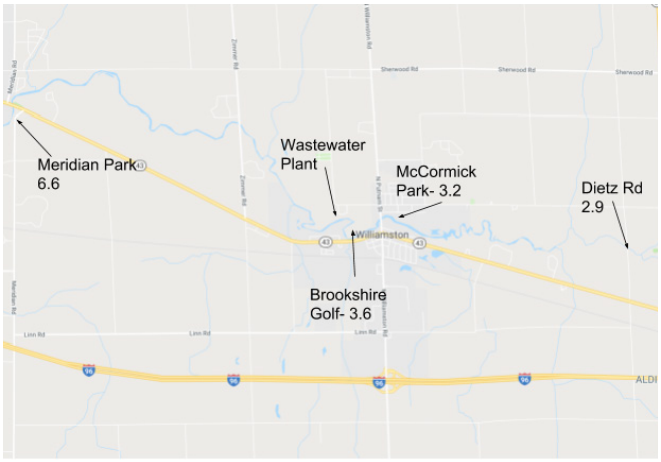


Figure 1: Map of Testing Sites. All of the values are the concentration, in microplastics per liter, at each site. We did not test the water directly outside of the wastewater plant, but included it to show where it is in correlation to the other sites.

can generally be categorized into five main types: fibers, beads, films, foams, and fragments (8). A fiber is a long strand of microplastic that is found in synthetic clothing and cigarette butts. A bead is a small pebble-like microplastic that is found in toothpaste and shampoo. A film is a small fragment of wrappers and plastic bags. A foam comes from styrofoam. Fragments are small pieces of plastic that have been chipped off plastic litter such as water bottles. We hypothesized that the majority of the plastics we found would be fibers. This is because a 2016 study of the Great Lakes found that around 70% of microplastics were fibers (8).

RESULTS

The foundation of this study was built on the hypothesis that we would see increases in microplastic levels as we looked further downstream, if we discovered any at all. Starting at 2.9 microplastics per liter by the Dietz Road Bridge (n= 3, SE= 1.1) the number of microplastics per liter increased steadily, peaking at Meridian Park (6.6 microplastics per liter, n= 2, SE= 5.8), the furthest site that we sampled downstream. At the other sites that we sampled, we found 3.2 microplastics per liter at McCormick Park (n=3, SE= .512) and 3.6 microplastics per liter at Brookshire Golf Course (n=3, SE=.864) (**Figure 2**).

One of our overarching research questions was "Does Williamston add microplastics pollution to the Red Cedar?" Based on our data it seems as though Williamston does add a substantial amount of plastics to the Red Cedar, but according to the Mann Whitney tests we performed, that does not seem to be the case. We found that the microplastics concentration was not significantly increased in the river as it passed through Williamston because our calculated U Value was 1.

Another aspect of our research dealt with the individual types of microplastics. In our samples from the river, we found fibers (91%), fragments (7%), beads (1%), foams (0.5%), and films (0.5%) (**Figure 3**). We also tracked the types of plastics that we found from the wastewater plant, where we found fibers (90%), fragments (6%), foams (2%), films (2%), and zero beads (**Figure 4**). Given this similarity in concentrations of the microplastic type, we decided to conduct a statistical analysis to see if the wastewater plant was the main emitter of microplastics into the Red Cedar River.

As previously mentioned, one of our initial hypotheses was that the Williamston Wastewater Treatment Plant would be the main reason for the increase in microplastics throughout Williamston. With the aid of Dr. Kurt Guter, an expert in water research, we used a dilution equation in order to test this.

After calculating the dilution by taking into account the average stream flow of the Red Cedar River and the average daily effluent of the wastewater treatment plant, we got a dilution factor of 127.4. This means that for every increase of 1 microplastic per liter downriver of the wastewater treatment plant, there should be 127.4 microplastics per liter in our wastewater samples if the wastewater treatment plant is the sole contributor of plastics. This poses a challenge to our initial hypothesis because we found only 6 microplastics per liter (n= 4, SE= .38) in the wastewater samples (**Figure 4**).

A final aspect of our research pertained to microplastics in drinking fountain water at Williamston High School. Midway through our testing period, Williamston experienced a 100-year flood that prevented us from sampling from the Red Cedar River. We used this time to sample water from the high school drinking fountains. We found plastics in all four drinking fountains that we sampled. We found our highest concentration of plastics at the 20-year-old D Hallway Fountain, where there were 4.4 plastics per liter (SE= 1.54). The lowest concentration of plastics that we found was at the 20-year-old Lunch Room Fountain, where we found 1.8 plastics per liter (SE= 1.476). This seems to be a very large difference, but it was not statistically significant because the ranges of the respective standard errors overlap (Figure 5).

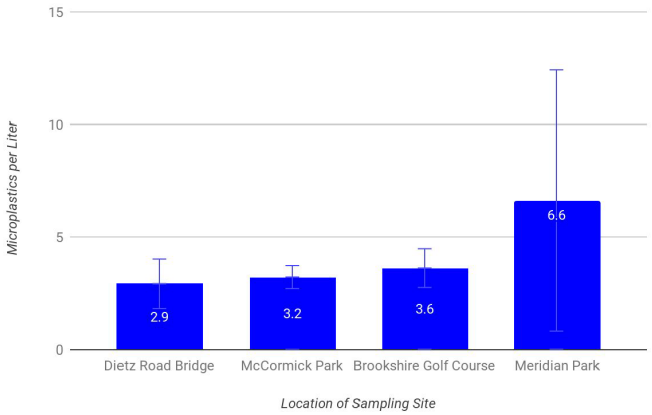


Figure 2: Microplastics Concentrations in the Red Cedar: Upstream to Downstream. The column values show mean concentration of microplastics and the error bars show standard error.

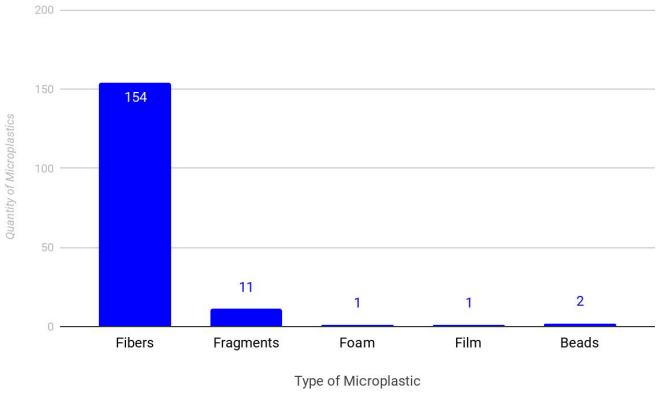


Figure 3: Total Microplastics by Type. The columns represent the totals of each microplastic found across all four river testing sites. These represent the total amount across all of our testing days and are not an average.

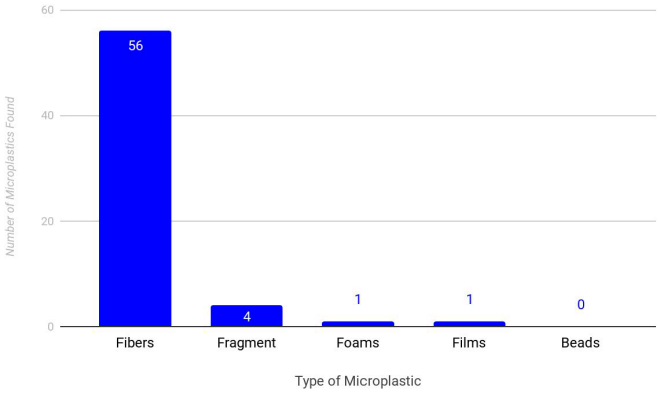


Figure 4: Wastewater Microplastics Totals by Type. The columns represent the total amount of each microplastic found at the Williamston Wastewater Treatment Plant. These columns represent total amount across all of our wastewater samples and do not represent averages.

DISCUSSION

Initially, most of our research questions were quantitative, concerning the concentration of microplastics in the Red Cedar within the Williamston School District. We found that throughout the river, levels of plastic debris increased from upstream to downstream. From our results, we see an increase in microplastics concentrations between the Brookshire Golf Course and Meridian Park.

However, a word of caution must be added to this conclusion. We did not have a large enough sample size to indicate significant results and there could be other factors at play that would cause an increase. Our results indicate an increase, but more testing must be done in order to achieve more conclusive evidence.

An important secondary aspect of our project concerned the source of these plastics. As suspected, the Williamston Wastewater Treatment Plant fits the criteria for a major source of microplastics in the Red Cedar. It lies between our testing sites of Brookshire and Meridian where we saw the most significant increase in pollution (**Figure 1**). Our hypothesis was that we would see extremely high levels of plastics in the outflow of the plant. However, according to our dilution calculation in the results, since the wastewater treatment plant is emitting only 6 plastics per liter, and not 127, it is not contributing nearly enough microplastics to the Red Cedar to fully explain the increase in microplastics from Brookshire to Meridian Park. Therefore, we concluded that the wastewater treatment plant may be contributing microplastics into the Red Cedar, but it is more likely that there are other sources contributing more plastics. A plausible explanation is that many of the microplastics are coming from the individual homes along the Red Cedar River in between Brookshire and Meridian Park. Each use of a washing machine can create around 700,000 microplastic fibers (9). We hypothesize that since many of the homes between Brookshire and Meridian Park do not utilize a city sewer system, instead relying upon septic tanks, there may be infiltration into the Red Cedar from those septic tanks causing the greater values. This is merely speculation and more testing would be required in order to confirm this hypothesis. What we do know is that the levels of microplastics, and more specifically fibers, are higher at Meridian Park than they are at the Brookshire site and that these plastics are coming from somewhere. Further testing is required to determine a definitive source.

An important component of our research dealt with the types of microplastics. We tracked the number of each type because we felt this might help us locate the source of the plastics and we wanted to compare our numbers to those found by other studies. In our research, we found that 91% of our plastics were fibers. This elevated level of fibers was similar to another analysis of microplastics in the Great Lakes Basin, in which the US Geological Survey found that in Great Lakes Tributaries, 70% of collected microplastics were fibers (8). Another noteworthy result that the categorization of microplastics revealed was the near absence of beads. Only 1% of our collected plastics were beads. We find this interesting since there was a nationwide ban on the use of microbeads that was implemented in 2016 (8).

If it is indeed true that Williamston is adding microplastics to the Red Cedar River, the future may be concerning. Currently, the EPA's range of regulation in regard to microplastics has been minimal, meaning that if the Williamston Wastewater Treatment Plant is a source of a majority of the Red Cedar's microplastics, it has no economic incentive to attempt to reduce its output. Another worrying aspect of this issue is the prospect of public health concerns resulting from the proliferation of plastic fibers in areas of commercial fishing, in this case the Great Lakes. If fish are consuming these microplastics, it is only a matter of time until the microplastics work their way up the food chain (10).

Another aspect of our research dealt with microplastics in drinking water supplies. It was interesting to note that in our samples, we found no correlation between age of a drinking fountain and the concentration of microplastics. This suggests

that the plastics are coming from other places such as the pipes leading to the fountains, not the fountains themselves. Again, since we only took two samples, more research is needed in order to establish the veracity of these results. What is alarming about our result is how it is mirrored in other studies of US drinking water. A recent study that tested sites such as the EPA Headquarters and Trump Tower found that 94% of US drinking water samples contained microplastics (11). In this study, the researchers found an average of 9.6 plastics per liter in the drinking water, which was higher than our average of 3.14 plastics per liter. Despite no research to date being conducted on the effects of microplastic consumption on humans, it is definitely something that should be avoided. Even bottled water, which is considered by many to be safer than tap water, has been found to contain microplastics. A study of US bottled water found that 90% of bottles contained microplastics (12).

While our research was conducted to the best of our available resources, there is room for improvement in terms of methodology and design. One issue is that we collected our samples in plastic buckets. While we ran blanks to ensure that there was no exfoliation, we would recommend that future samples be collected in glass jars. Another area that could be improved relates to the filters. We used the best paper filters available to us and checked 50% of them before use for microplastics, only finding plastics on 2 occasions. However, we would recommend individually wrapped cellulose membrane filters in the future since these are the filters used by Dr. McNeish at Loyola University. Our final recommendation would be to collect more samples. We were not able to obtain statistically significant evidence due to our small sample size. We feel that such an issue could be averted with a larger sample size.

Additionally, our study faced certain limitations. The applications of our findings are limited to gaining an understanding of the microplastics problem in our community. Williamston's microplastics levels will not be representative of the microplastics levels in other areas due to certain factors such as population and industry. Additionally, our team sampled a relatively narrow section of the Red Cedar, seeing as we sampled within the Williamston school district, and only sampled four spots. Therefore, our results are only applicable to the portion of the Red Cedar River as it passes through Williamston.

In the interest of time and due to limited materials, we decided to confine our research to four sites in the Williamston area. This did not in any way hurt our overall research goal; in fact, these guidelines helped our research by providing a clear focus upon microplastics in Williamston. Another boundary of limitation of our research was that we only sampled water. We did this in the interest of time, since sediment separation (another method of sampling) takes far longer to analyze than bulk water separation. Another limitation of our research is time. We sampled in a 5-month window, and had we been able to sample over a longer span of time, we would be able to have a better idea of the average microplastics concentration in the Red Cedar River.

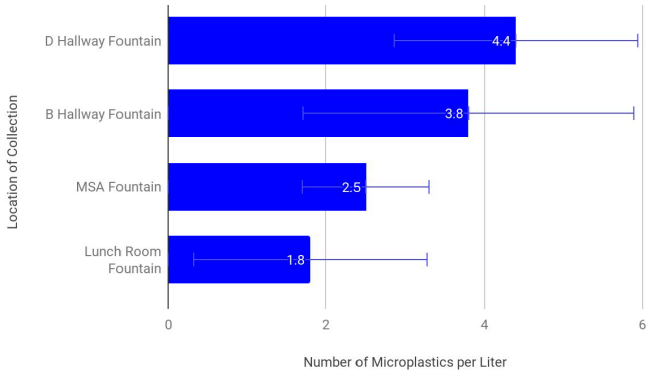


Figure 5: Microplastics in Williamston High School Drinking Fountains. The column values are means and the error bars show standard error.

Overall, we had some surprising results and some expected results. The quantity of microplastics that we saw was expected since it fell within the 0-10 microplastics per liter range that we hypothesized that we would see. However, we were surprised that the data we collected suggested that we were incorrect in our assumption that the wastewater treatment plant would be the primary emitter of microplastics into the Red Cedar. Since we know the wastewater plant is not the source of the pollution, and using this information we could develop another hypothesis that states that septic systems and washing machines are the primary polluters of microplastics into the river. Our findings pose an interesting challenge to our community, both in regards to how to deal with the pollution, but also how to find the definitive source and how to end the pollution.

MATERIALS AND METHODS

Site Selection

In our study, we analyzed the levels of microplastic concentration in Williamston's waterways. We primarily tested the Red Cedar River, but we also tested the final effluent from the Williamston Wastewater Treatment Plant. We tested the microplastics levels by collecting bulk water samples from the Red Cedar. The bulk water was collected by submerging a five-gallon bucket into the river. We filled the buckets with approximately 2.5 gallons of river water of each sample.

We collected samples from four sites along the Red Cedar. These sites included the location where the Red Cedar enters the Williamston School District at Dietz Road, McCormick Park, the 12th hole at Brookshire Golf Course, and the location where it leaves the school district at Meridian Road. These locations were chosen to show how much the Williamston area as a whole is contributing to the microplastics problem. At each location, we sampled in the exact same spot in order to maintain consistent results. We also sampled each site two to four times (November 2017- April 2018) in order to have a more representative set of data.

We also took samples from the Williamston Wastewater Treatment Plant. The manner in which we sampled from the wastewater treatment plant was slightly different than the manner in which we sampled from the river. We collected a two-gallon sample from a composite 24-hour sample from the wastewater treatment plant. We used a composite sample of the final effluent because we wanted an accurate representation of how many microplastics the plant was emitting, and if we used a sample from a specific point in time, it would be subject to variation in the quantity of microplastics at that given time.

We also sampled from one off-river site; this was the Williamston High School building. While scanning for our literature review, we came upon an article that stated that over 90% of tap water samples in the United States contained microplastics. We tested this in our high school by collecting two separate 1 liter samples from four drinking fountains throughout the school. We then ran them through the same process as the river samples.

Sample Processing

We used a systematic process to gather data from our samples. Once we collected the bulk water samples, we brought them to the lab in order to filter them. We used gridded filter paper to filter the water from the microplastics. We attempted to control the infiltration of microplastics by examining 50% of the filters for potentially misleading particles prior to using them. We then used an aspirator vacuum in order to suck the water through the filter. The filtered water was placed into a beaker to determine the exact quantity of water that passed through the filter. Once the sample was filtered, we placed the filter paper into an incubator for 48 hours at 60°C to dehydrate the sample and remove all the water from the filter, thus making it easier to analyze under a microscope (13). After a 48-hour period, we put the filter into a petri dish and scanned for microplastics under 10x magnification (14). The grid on the filter paper helped us to scan the sample systematically. After both group members agreed on the number of microplastics on a given filter, we organized the data by splitting the different particles into categories based on the type of microplastic.

There were a few materials that we needed in order to accurately complete our research. The primary materials that we needed were an aspirator vacuum, paper filters, an incubator, a dissecting microscope, and glass beakers in a variety of sizes. We were provided with a materials list from Dr. Rachel McNeish (an expert in microplastics at the University of Loyola Chicago) and found that no other tools were required.

Statistical Analysis

Finally after we conducted all of our sample processing, we performed a variety of statistical analyses, namely dilution equations and Mann-Whitney U Tests. We used a one-tailed Mann Whitney test because this test does not make assumptions about standard deviation and is more applicable to our research findings. To determine if there was statistical evidence that Williamston adds microplastics, we used a null hypothesis that the concentration of microplastics at Dietz Road would be the same as at Meridian Park. Our alternate hypothesis was that the concentration at Meridian Park would be higher. We also conducted a dilution calculation when we were evaluating our hypothesis that the Williamston Wastewater Treatment Plant was the source of Williamston's microplastics pollution.

ACKNOWLEDGEMENTS

In order to complete our research, we needed some help from outside sources. Dr. Kurt Guter provided us access to the wastewater treatment plant's final effluent. He was also instrumental in focusing the scope of our research. Under his guidance, we were able to overcome several obstacles, including minimizing the risk of allowing unwanted microplastics to get onto our filters. We also consulted Dr. Rachel McNeish from Loyola University whose input we relied upon throughout the early stages of the research process. She provided us with our methodology, and we couldn't have conducted our research without her.

Received: June 14, 2018
Accepted: January 9, 2019
Published: February 11, 2019

REFERENCES

1. Nahigyan, Pierce. "By 2050, There Will Be More Plastic in the Ocean Than Fish." Planet Experts, 27 May 2016, www.planetexperts.com/by-2050-there-will-be-more-plastic-in-the-ocean-than-fish/.
2. Katz, Brigit. "Dead Sperm Whale Had 64 Pounds of Trash in Its Digestive System." Smithsonian.com, Smithsonian Institution, 12 Apr. 2018, www.smithsonianmag.com/smart-news/dead-sperm-whale-had-64-pounds-trash-its-diges tive-system-180968776/.
3. Mani, Thomas, et al. "Microplastics Profile along the Rhine River." Scientific Reports, vol. 5, no. 1, 2015, doi:10.1038/srep17988.
4. What Do We Know Today About Microbeads and Microplastics in the Ocean?" NOAA, 2015, response.restoration.noaa.gov/about/media/what-do-we-know-today-about-microbeads-and-microplastics-ocean.html.
5. Baldwin, Austin K., et al. "Plastic Debris in 29 Great Lakes Tributaries: Relations to Watershed Attributes and Hydrology." Environmental Science & Technology, vol. 50, no. 19, 2016, pp. 10377–10385., doi:10.1021/acs.est.6b02917.
6. Castañeda, Rowshyra A., et al. "Microplastic Pollution in St. Lawrence River Sediments." Canadian Journal of Fisheries and Aquatic Sciences, vol. 71, no. 12, 2014, pp. 1767–1771., doi:10.1139/cjfas-2014-0281.
7. Jerome, Sara. "Wastewater Plants Linked To Microplastic

Pollution." Www.wateronline.com, 2018, www.wateronline.com/doc/wastewater-plants-linked-to-microplastic-pollution-0001.
8. "Microplastics in Our Nations's Waterways." U.S. Water Use, United States Geological Survey, 2016, owi.usgs.gov/vizlab/microplastics/.
9. Paddison, Laura. "Single Clothes Wash May Release 700,000 Microplastic Fibres, Study Finds." The Guardian, Guardian News and Media, 27 Sept. 2016, www.theguardian.com/science/2016/sep/27/washing-clothes-releases-water-polluting-fibres-stud y-finds.
10. Weikle, Brandie. "Microplastics Found in Supermarket Fish, Shellfish | CBC News." CBCnews, CBC/Radio Canada, 28 Jan. 2017, www.cbc.ca/news/technology/microplastics-fish-shellfish-1.3954947.
11. Carrington, Damian. "Plastic Fibres Found in Tap Water around the World, Study Reveals." The Guardian, Guardian News and Media, 5 Sept. 2017, www.theguardian.com/environment/2017/sep/06/plastic-fibres-found-tap-water-around-world-study-reveals.
12. Readfearn, Graham. "WHO Launches Health Review after Microplastics Found in 90% of Bottled Water." The Guardian, Guardian News and Media, 15 Mar. 2018, www.theguardian.com/environment/2018/mar/15/microplastics-found-in-more-than-90-o f-bottled-water-study-says.
13. Kim, Lisa, et al. "Analysis of Microplastics in Water and Sediment" , 2016, https://docs.google.com/document/d/1QZfXKn0JWN2sQ6tSxu-evSDdOdiD7k5JRxFr2bcQUms/edit?usp=sharing
14. Barrows, A. "Guide to Microplastic Identification." Marine & Environmental Research Institute, 2017, www.ccb.se/documents/Postkod2017/Mtg050317/Guide%20to%20Microplastic%20Iden tification_MERI.pdf

JOURNAL OF
EMERGING INVESTIGATORS

Article

Phages can be more effective and specific than antibiotics in combating bacteria

Leon L. Wu and Gwyneth M. Pinta
Upper Arlington High School, Upper Arlington, OH

SUMMARY

Every year tens of thousands of people die of infections from antibiotic-resistant bacteria. Phage therapy has been suggested as an alternative because bacteria resistant to antibiotics may still be susceptible to phages. Furthermore, phages have the advantage of being more specific and thus less likely to harm beneficial bacteria. However, phages may have limited effectiveness in combating bacteria since bacteria possess several antiviral defense mechanisms and can quickly develop resistance to phages. The purpose of this study was to compare the effectiveness and specificity of antibiotics and phages in combating bacteria. To this end, we exposed strains of the bacteria Escherichia coli to T4 bacteriophages and antibiotics and assessed effectiveness and specificity of bacterial killing. Fission yeast, a unicellular eukaryotic organism, was used as a control. Cell growth, phage plaque formation, or cell concentrations were observed or measured. It was found that T4 phages are more specific and effective in fighting or inhibiting both antibiotic-resistant and sensitive bacteria than antibiotics. One phage can kill at least 4000 actively growing bacterial cells within two hours. Therefore, the data suggest that phage therapy can be developed as an efficient tool to combat antibiotic-resistant bacteria.

INTRODUCTION

Due to the overuse of antibiotics in the healthcare and food industry, antibiotic resistance in bacteria has become a global problem. Every year, approximately 700,000 people die from antibiotic-resistant infections worldwide (1). If this trend continues, by 2050, a projected 10 million people will have died from antibiotic resistant infections at a cost of $100 trillion unless new and effective treatments are quickly developed (1). In the U.S. alone, tens of thousands of deaths are annually attributed to drug-resistant strains of the bacterium Escherichia coli (2). Some bacteria, such as Neisseria gonorrhoeae and Staphylococcus aureus, have developed resistance to essentially all classes of antibiotics (3-5). Unfortunately, very few new classes of antibiotics have been recently discovered and approved (3, 6, 7).

Most E. coli strains (**Figure 1A**) are non-pathogenic bacteria that inhabit the intestines and normally aid with food digestion. However, pathogenic E. coli with virulence factors can be contracted from a variety of fairly common sources. E. coli can be found in improperly sanitized produce, unpasteurized liquids, and contaminated meats (8). Even healthcare equipment, such as endoscopes, has also led to some outbreaks of E. coli superbugs in hospitals. The rapid growth rate is one reason why a bacterial infection can be so dangerous. When provided with optimal growth conditions (such as nutrients and optimal temperature), E. coli cells are capable of doubling their population every 20 minutes (9). At this alarming rate, a single E. coli cell can produce 4,722,366,482,869,645,213,696 (272) progeny in a single day. Bacterial pathogens can still proliferate very fast even in people with healthy immune system, so many infections need to be treated urgently.

E. coli has led to many deaths; the H30Rx E. coli superbug alone has resulted in thousands of deaths in the United States. Drug resistance in E. coli is steadily rising especially for antibiotics such as the commonly prescribed fluoroquinolones and certain generations of cephalosporin (10). In addition, an increasing number of strains are also showing resistance to antibiotics like ampicillin, penicillin, and streptomycin (11). Both classical E. coli pathogens and commensal opportunistic E. coli strains can become antibiotic-resistant. The H30Rx strain, a clinical isolate, was identified twenty years ago when a strain of E. coli developed mutations resulting a new strain, H30R, which is resistant to the antibiotic Ciprofloxacin (12). This in turn paved the way for H30Rx, which is resistant to several antibiotics (12). H30Rx also has the capability to disseminate into the bloodstream from urinary tract infections causing systemic inflammation, which can become life-threatening (12). Unfortunately, H30-Rx is not the only deadly superbug that exists. Carbapenem (a last resort antimicrobial)resistant bacteria has a ~50% mortality rate according to one report (13).

Due to the dramatic uptick in drug resistance, many people have suggested using phage therapy as a solution to combat bacteria (1, 14). Phage therapy is a method in which bacteriophages (or phages) are used to kill pathogenic bacteria (14). Phages, discovered a century ago, are viruses that lyse and kill bacteria (**Figure 1B**). They are found everywhere on the earth and an estimated 30 billion phages are absorbed into our bodies via our intestines each day (15). Phages are essential to maintaining a healthy microbial ecosystem in human digestive tracts. They were mentioned as a tool for curbing antibiotic resistance threats

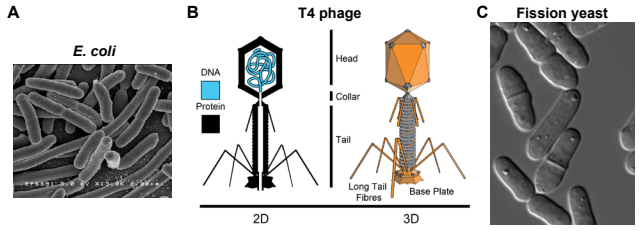


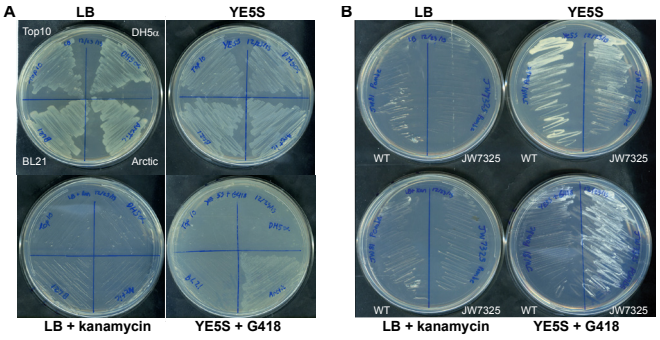
Figure 1. Images of E. coli (A), T4 phage (B), and the fission yeast S. pombe (C). The images of E. coli (Credit: Rocky Mountain Laboratories, NIAID, NIH) and T4 phage (Credit: authors Adenosine and Pbroks13) are from Wikimedia Commons.

in a 2014 status report by the National Institute of Allergy and Infectious Diseases (16). Phages have been safely used to treat bacterial infections since the 1920's, but they fell out of favor due to the discovery of antibiotics. However, there is now a renewed interest in phage therapy thanks, in part, to escalations in antibiotic resistance and the relative safety of phages. For example, Listex, a phage based solute used to kill Listeria and Salmonella in foods, was approved by the FDA in 2006 (17, 18). UCSD psychologist Thomas Patterson, who was infected with a bacterium resistant to all the available antibiotics, and five other people were successfully cleared of bacterial infections with phage cocktails by a UCSD team (19). Proponents believe that phage therapy is more precise than antibiotics, which are typically broad-spectrum and kill or inhibit both pathogenic and beneficial bacteria, since most phages specifically target certain species of bacteria. Due to this specificity, phages also cause less side effects in humans. Despite these advantages and the recent successes, phage therapy is still rarely used in the US and still has several obstacles to overcome. Research has shown that certain phages can help transfer drug resistance and lysed bacterial cells may release toxins (20). Moreover, bacteria also have several antiviral mechanisms to protect themselves from phages, such as the famous CRISPR/Cas defense systems (21-24). Therefore, bacteria may eventually develop immunity to phages. This will be problematic if more and more people decide to use phages over antibiotics. However, unlike antibiotics, phages may be able to evolve alongside these bacterial pathogens, avoiding some of the issues with resistance.

Here, we tested the specificity and effectiveness of traditional antibiotics compared with phages on limiting growth of E. coli. Fission yeast (**Figure 1C**), a genetically tractable unicellular model organism with cell-division cycle and many proteins/genes conserved in human cells (25-28), was used as a eukaryotic control. Wildtype fission yeast cells are sensitive to many antibiotics including geneticin (G418), hygromycin, and nourseothricin (clonNAT) (29). We compare the efficiency and specificity of T4 phage and several antibiotics in inhibiting or killing yeast and E. coli strains on agar plates or in liquid culture. It was hypothesized that T4 phages are more effective than antibiotics since they will be able to target and infect antibiotics-resistant bacteria. In addition, phages will remain active resulting from their proliferation even after the antibiotics are depleted, so this may also make them more effective. Indeed, we find that T4 phage is more specific and efficient in killing both antibiotic-sensitive and antibiotic-resistant bacterial cells. We predict that therapies employing both phages and antibiotics will be ideal to combat antibiotic-resistant bacteria.

RESULTS

First, we tested the specificity of antibiotics by observing the viability and growth of bacteria and fission yeast on LB (standard medium for bacteria) and YE5S (rich medium for fission yeast) plates with broad spectrum antibiotics kanamycin or geneticin (G418), respectively. Kanamycin binds to the 30S subunit of prokaryotic ribosomes and interferes with protein synthesis. It is effective in treating severe bacterial infections including tuberculosis, but it may have side effects including hearing and kidney problems. G418 inhibits the elongation step of protein synthesis in both prokaryotic and eukaryotic cells. As shown in **Figure 2**, A and C, E. coli strains Top10, DH5α, BL21, and ArcticExpress were inviable in LB medium with kanamycin, unlike the normal growth in LB medium without kanamycin (**Figure 2A**, left). The only strain that grew well on the medium with G418 was the ArcticExpress cells (**Figure 2A**, right). Thus, all the tested E. coli strains are sensitive to kanamycin and G418 except ArcticExpress cells, which is resistant to G418 (**Figure 2C**). Fission yeast cells did not grow well on LB medium even without kanamycin (**Figure**



C

Medium	Top10	DH5α	BL21	Arctic	WT yeast	JW7325
LB	+	+	+	+	?	?
LB + kanamycin	-	-	-	-	?	?
YE5S	+	+	+	+	+	+
YE5S + G418	-	-	-	+	-	+

Figure 2. Antibiotics can kill both prokaryotic E. coli cells and eukaryotic fission yeast cells. (A) E. coli strains Top10 (top left), DH5α (top right), BL21 (bottom left), and ArcticExpress (bottom right) as marked were re-streaked onto LB, LB + kanamycin, YE5S, YE5S + G418 plates and grown at 37˚C for 1 day before scanning. (B) Fission yeast strains wildtype (WT; left) and JW7325 (right) were re-streaked onto LB, LB + kanamycin, YE5S, YE5S + G418 plates and grown at 25˚C for 2 days before scanning. Two replicates were performed for each experiment. (C) Summary of antibiotic sensitivity of E. coli and fission yeast cells as shown in (A) and (B). +, growth or resistant to the antibiotic; -, no growth or sensitive to the antibiotic; ?, unknown due to poor growth of yeast on LB medium.

2B, left), but YE5S + kanamycin plates were not available. Due to these limitations, it was difficult for us to determine if the yeast cells are sensitive to kanamycin or not (**Figure 2C**). However, we confirmed that yeast strain JW7325 (kanMX6-Pypt3-tdTomato-ypt3) but not wild-type yeast is resistant to G418 (**Figures 2B** and **2C**). Taken together, this data confirms that an antibiotic can target and kill many different kind of cells.

Next, we tested the specificity of the T4 phage by observing phage plaque formation on lawns of bacteria and fission yeast. Plaques are formed on lawns of cells when they are lysed and cleared by phages. Both antibiotic-sensitive and resistant E. coli strains were susceptible to being lysed by T4 phages, as evidenced by the various sizes of phage plaques (cleared zones without bacteria) with different amount of phages (**Figure 3A**, examples marked by arrows). In contrast, no phage plaques were formed on the lawns of either G418-sensitive JW81 or resistant JW7325 fission yeast cells (**Figure 3B** and data not shown). Thus, it can be concluded that the T4 phages are more specific than antibiotics because they cannot infect yeast while antibiotics kills both bacteria and yeast.

Then, we investigated the efficiency of how phages attack and lyse bacterial cells. Ten-fold serially diluted phages were added to 300 µl DH5α cells (with OD600 = 0.75; ~1.8 x 108 bacterial cells given that 1 OD600 = 8 x 108 cells/ml), incubated for ~1 hour, and then plated on agar plates. After incubation at 37˚C overnight, more phage plaques were formed with increasing concentrations of phages (**Figure 4A**). At 10-3 dilution of T4 phages (4 x 109 phages/ml x 10-3 x 10 µl x 10-3 ml/µl = 40,000 phages), ~90% area of the plate had no or significantly reduced DH5α cells (**Figure 4A**). Thus, one phage (from the starting stock) can kill ~4000 bacterial cells (~1.8 x 108 bacterial cells x 90% / 40,000 phages), which is highly efficient. Interestingly, at 10-2 phage dilution (400,000 phages/per plate), approximately hundreds to thousands of single bacterial colonies were formed on each plate (**Figure 4A**), which suggests that phage-resistant cells can quickly take over the culture once the sensitive bacteria die off. At 10-3 dilution of T4 phages, phage-sensitive cells may have used up the nutrients before they died so that the resistant cells have no chance to proliferate and form colonies. The results were confirmed using BL21 cells (**Figure 4B**). Together, it was determined that T4 phages are highly efficient at killing bacterial cells, but resistance to phages can also develop or be selected quickly.

Lastly, we directly compared the effectiveness of killing/inhibiting bacteria between phages and antibiotics in terms of their weights. The molecular weights of ampicillin, G418, and T4 phages are 371, 693, and 1.93 x 108 g/molar (30), respectively. In this experiment, G418-resistant but ampicillin and kanamycin-sensitive ArcticExpress E. coli cells were mixed with either phages or antibiotic ampicillin or G418 at time zero. Cells were grown at 37°C and OD600 was measured every 30 minutes (**Figure 5A**). We found that the phages were highly effective in killing bacteria compared to the antibiotics. Phages at concentrations of both 103 dilution (phages : bacteria = 1 : 4,500) and 102 dilution (phages : bacteria = 1 : 450) lysed essentially all the E. coli cells after a 30 to 60 minutes delay as the OD600 of bacterial cells increased initially but then dropped to almost zero and the culture became clear in about 90 minutes. This confirms

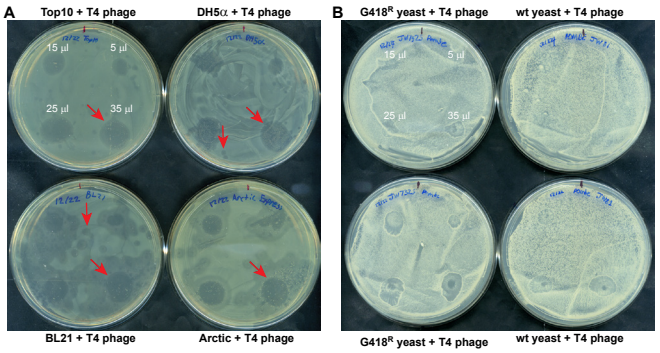


Figure 3. T4 phage can lyse E. coli cells but not fission yeast cells. (A) Formation of phage plaques (examples marked with arrows) on LB plates with fresh lawns of E. coli cells, which were inoculated with 5, 15, 25, and 35 µl (counterclockwise starting from top right as shown on the first plate) undiluted T4 phages (4 x 109 phages/ml) for each strain and incubated at 37˚C. (B) Insensitivity of wildtype fission yeast JW81 and G418-resistant JW7325 (G418R) stains to T4 phages. Fresh lawns of yeast cells on YE5S plates were inoculated with 5, 15, 25, and 35 µl (counterclockwise starting from top right as shown on the first plate) undiluted T4 phages for each strain and incubated at 36˚C. Images from two repeated experiments were shown. On some plates yeast cells were displaced by the phage solution, as evidenced by more cells on the edge of the spots with phages. Similar experiments were performed for four times.

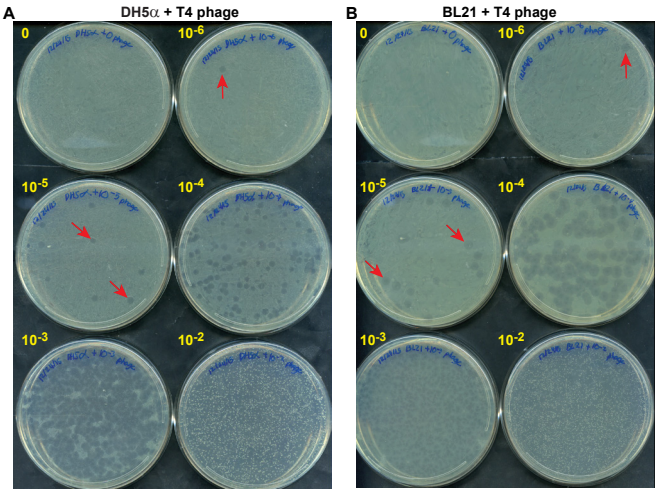


Figure 4. T4 phages efficiently lyse actively proliferating bacterial cells. Active growing DH5α (A) and BL21 cells (B) were mixed with 10 µl different dilutions of T4 phages as indicated, incubated for ~1 hour, and then plated evenly on plates, and incubated at 37˚C overnight before scanning. Examples of phage plaques were marked with arrows. Three replicates were performed for each experiment.

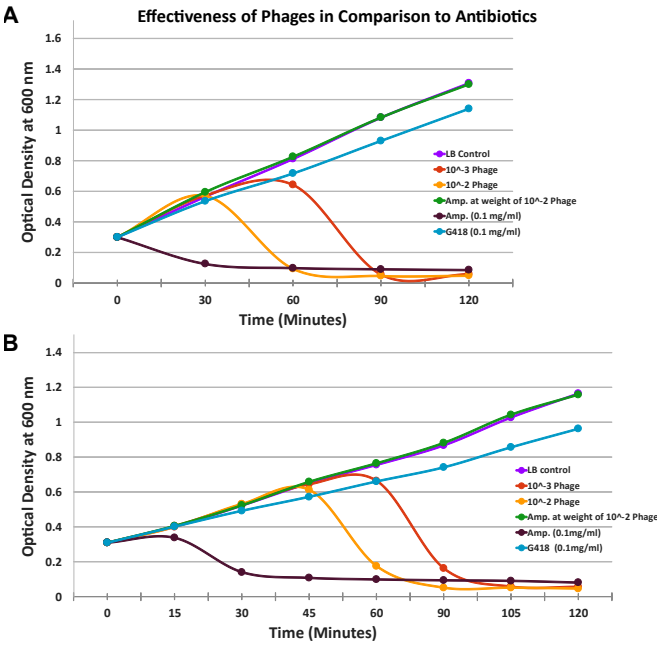


Figure 5. Phages are more effective than antibiotics in killing or inhibiting bacteria. Density of ArcticExpress cells treated with different concentrations of T4 phages and antibiotics (or 40 µl LB medium as control) was measured every 30 (A) or 15 minutes (B) and plotted using Excel. The experiment was performed twice.

that a phage can kill >4000 actively growing bacterial cells within 2 hours. Ampicillin added with a weight equivalent of the 102 phage dilution had no effect on bacterial growth. The ampicillin stock solution was functional since 0.1 mg/ml of ampicillin (normal lab working concentration, which is 590,000 times higher than 102 phage equivalent) quickly inhibited cell growth in ~30 minutes. As expected, 0.1 mg/ml of G418 only mildly slowed cell growth. The experiment was repeated by measuring the OD600 every 15 minutes. Essentially identical results were obtained (**Figure 5B**). Thus, the T4 phage is much more effective in killing G418-resistant bacteria than ampicillin if applied at the same weight, although each phage is 520,000 times heavier than one ampicillin molecule. Taken together, our data indicate that T4 phage is more specific and effective than antibiotics in killing both antibiotic-sensitive and resistant-bacteria.

DISCUSSION

In this study, we found that the T4 phage is significantly more effective than antibiotics in fighting bacteria if measured by their weight, even though a phage is 520,000 times heavier than an ampicillin molecule and thus much less was used in terms of molar concentration, which makes phages much more potent (Figure 5). This is partly because the phages continue to infect bacteria and proliferate even after antibiotics have already been degraded or depleted. Phages are also more selective than antibiotics since they only target one or a few specific types of bacteria via cell surface receptors, not fungal or human cells. This is confirmed because they do not kill eukaryotic yeast, despite the fact that G418 inhibits growth of wildtype yeast cells. Moreover, the T4 phage is also effective against G418-resistant bacterial strain ArcticExpress. Thus, our data support the hypothesis that phages are more effective and specific than antibiotics in combating bacteria.

In the future, a study can be conducted using phages and antibiotics in conjunction to test if both antibiotic-resistant and phage-resistant bacteria will be eliminated. Moreover, the ED50 (median effective dose) for phages should be measured in future studies. Limitations of this study were the sample size and availabilities of materials. The effect of phages on bacterial populations in liquid cultures was only done twice. However, since both trials produced nearly identical results, an increase in trials may not yield any differences. No YE5S + kanamycin plates were available to test if yeast cells are sensitive to kanamycin. Moreover, we did not have access to tissue culture cells so that we could not test the effectiveness and specificity of phages and antibiotics in treating bacterially-infected human cells.

Phages are crucial to the global ecosystem and human microbial community by regulating bacterial abundance, balance, and diversity (31). For example, it was estimated that there are >1031 phages on earth and that one third of marine surface bacteria are wiped out by phages every day (32). In a given ecosystem, approximately 80% of bacterial death is caused by phage infection (31). Phages are enriched >4 times in our mucus layers compared to the adjacent environment (15). Billions of phages enter human tissues via our intestines each day and they may modulate our immune system (15). Thus, it is possible to harness this powerful control on bacterial populations by phages and use it to help fight bacterial infections with minimal side effects. In fact, phages or phage cocktails have already been shown to be successful in treating chronic infections (33), antibiotic-resistant bacterial biofilms (34), and multi-drug resistant bacterium Acinetobacter baumannii (19). In general, phage cocktails contain a variety of phages, each with different host targets, which ensure their effectiveness, minimize the development of phage resistance, and lower the risk of harming patients' microbiome (31). Engineered phages with a broader spectrum and minimal host immune reactions will thus be more effective and should be developed urgently. Ideally, phage banks with wildtype and engineered phages against all known bacterial pathogens should be established at hospitals. Bacterial pathogens from patients can be identified within hours by rapid DNA sequencing using powerful sequencers. Then the corresponding phages will be administered immediately to cure or save patients from bacterial infections. Because phages can only reproduce inside bacteria, the phages will stop proliferating once pathogens are cleared and may be eliminated by our immune system over time. Thus, the administered phages should not cause many side effects.

Our research provides useful insights into the potential

of phage therapy and could help design better treatments for many diseases caused by antibiotic-resistant bacteria. We found that one T4 phage can kill >4000 bacterial cells in 2 hours. This result challenges the conclusion from previous studies that significant numbers of phages (or huge phage-to-bacterium ratio) are needed to efficiently combat bacteria such as E. coli (35, 36), which is one of main obstacles for phage therapy. Actually, phage resistance may develop faster if the phage concentration is too high, as shown in Figure 4. Our data also suggest that phages are most effective in killing actively growing bacteria when the two are mixed together. Thus, the timing of phage delivery to bacteria is important. One disadvantage of phage therapy is that endotoxins (more likely to be released by lysing bacteria) may trigger immune responses such as fever or toxic shock in some patients. However, phages have much less overall side effects and thus higher therapeutic index than antibiotics because they only infect/target bacteria and archaea but not eukaryotes such as yeast and humans (33). Phages can be used to treat multiple-drug resistance bacteria, which cause severe problems for patients with suppressed or compromised immune systems and threaten to return humankind to the era before antibiotics (37, 38). Antibiotics, besides their well-known side effects, have the disadvantage of killing both pathogenic and beneficial microflora, which may disrupt the microbial balance and trigger severe secondary infections and allergic reactions in humans. In addition, commonly prescribed antibiotics such as Cipro and Levofloxacin have disabling side effects in some people whose mitochondria may be sensitive and damaged by the antibiotics, since mitochondria were evolved from symbiotic bacteria-like cells (39). However, no evidence shows that phages target or damage mitochondria since mitochondria have no bacterial surface receptors targeted by phages. Moreover, while it may take several years to develop a new antibiotic, phages are capable of adapting to phage-resistant bacteria within a few weeks (38). Thus, phage therapy is a fluid form of treating bacterial infections because it could evolve and be selected alongside bacteria as a flexible solution to combat resistance. Thus, many diseases, such as sepsis, could soon have more treatment options.

Despite the greater effectiveness phage therapies are still limited by resistant strains of bacteria. This problem could be addressed if antibiotics and phages are used simultaneously to kill the resistant strains. Lower doses of antibiotics may be enough if applied with phages. Indeed, it has been shown that phages can restore antibiotic sensitivity in multi-drug resistant Pseudomonas aeruginosa by using its multidrug efflux systems as receptor-binding site and phages can suppress bacterial immune system by expressing anti-CRISPR proteins (40, 41). More efforts and studies should be launched to improve phage therapy so that we will be able to combat the ever increasing threat of antibiotic resistance.

MATERIALS AND METHODS

Bacterial and Fission Yeast Media and Strains

Standard bacterial and yeast media were used. LB medium (984 ml ddH2O, 10 g Bacto tryptone, 5 g Difco yeast extract, 10 g NaCl, and 100 µl 10 M NaOH for one liter medium; plus 15 g agar for plates) was used for bacteria. Antibiotics ampicillin and kanamycin were used at final concentrations of 100 mg/L and 50 mg/L, respectively. YE5S medium (980 ml ddH2O, 5 g Difco yeast extract, 30 g dextrose, and 18 g agar for one liter medium) with or without 100 mg/L G418 (geneticin) was used for the fission yeast S. pombe. Non-pathogenic versions of bacterium E. coli (**Figure 1A**) strains used were: ArcticExpress RIL (230193, Agilent Technologies), BL21 (69451, Novagen), Top10 (Invitrogen), and DH5α (Invitrogen), which were generous gifts from the Wu lab at The Ohio State University.

The T4 phage (**Figure 1B**) was purchased from Carolina Biological Supply Company (item # 124335) with a titer of 4 x 109 plaque-forming particles/ml and stored at 4˚C before use. We simply assumed that a plaque-forming particle represented a phage in this study.

Fission yeast (**Figure 1C**, the image was taken using a Nikon microscope) strains used were G418 sensitive strain wildtype strain JW81 (h- ade6-M210 leu1-32 ura4-D18) and G418 resistant strain JW7325 (h- kanMX6-Pypt3-tdTomato-ypt3 ade6-M210 leu1-32 ura4-D18) from the Wu lab collection.

Methods

Sterile techniques were used throughout the experiments to avoid contamination. These techniques included sterilizing the bench with 70% ethanol and turning on a Bunsen burner when handling cells. E. coli strains of ArcticExpress, DH5α, BL21, and Top10 and fission yeast strains JW81 and JW7325 were cultured from -80˚C storage. Bacterial cells were grown on LB plates at 37˚C and yeast cells on YE5S plates at 25˚C. The cells were then streaked onto plates with or without antibiotics using sterile tooth picks to test their sensitivity.

Three experiments were performed to test specificity and efficiency of the T4 phage in impeding yeast and E. coli cells. First, E. coli or yeast cells were inoculated into sterile culture tubes with 4 ml of LB or YE5S liquid medium, and then grown at 37 (E. coli) or 25˚C (yeast) overnight. A 300 µl aliquot of cells of each strain was placed on each LB or YE5S plate and spread evenly using sterile glass beads (3 mm in diameter and approximately 4 beads per plate). The plates were incubated at 37 or 25˚C incubator until a lawn of cells formed. Then different amounts (5, 15, 25, and 35 µl) of undiluted T4 phages (4 x 109 phages/ml) were placed onto bacterial or yeast lawns to observe phage plaque formation after further incubation at 37˚C (E. coli) or 36˚C (yeast). Second, 300 µl E. coli or yeast cells grown at exponential phase were mixed with different concentrations of T4 phages, incubate for ~1 hour, and then plated evenly on LB or YE5S plates using sterile glass beads and incubated at 37˚C (E. coli) or 36˚C (yeast). OD600 (optical density at 600 nm wavelength) of the liquid cultures was: 0.51

for yeast and 0.75 for DH5α and BL21 cells. The T4 phage stock was serially diluted by adding 10 µl phages to 90 µl of LB or YE5S liquid medium. Then the process was repeated with the resulting dilution to get a dilution series of 101, 102, 103, 104, 105, and 106. We added 10 µl diluted phages to 300 µl cells and spread cells using glass beads. Yeast plates were incubated at 25˚C and E. coli plates at 37˚C. The plates were scanned using Epson Perfection V350 Photo scanner. The area with cells was measured using ImageJ (National Institutes of Health). Third, ArcticExpress cells were grown in liquid LB medium in sterile flask at 37˚C to OD600 = 0.3. The culture was divided into 6 flasks with 30 ml in each flask. T4 phages, ampicillin, and G418 at indicated concentrations were added to each flask. Cells were grown at 37˚C. Samples were taken every 30 minutes to measure cell density at OD600 using a spectrophotometer (Beckman Coulter DU730). The experiments were repeated by measuring the OD600 every 15 minutes.

ACKNOWLEDGEMENTS

We would like to thank Kenneth Gerien at The Ohio State University for providing the necessary materials and technical help to complete this study. We also thank Dr. Bryan Wenger at Upper Arlington High School for the critical reading and review of the manuscript.

Received: March 8, 2018
Accepted: April 28, 2018
Published: February 18, 2019

REFERENCES

1. Lin et al. “Phage Therapy: An Alternative to Antibiotics in the Age of Multi-Drug Resistance.” World J Gastrointest Pharmacol Ther 8(3), 2017, pp. 162-173.
2. Ventola CL. “The Antibiotic Resistance Crisis: Part 1: Causes and Threats.” P & T 40(4), 2015, pp. 277-283.
3. Rossolini GM, Arena F, Pecile P, & Pollini S. “Update on the Antibiotic Resistance Crisis.” Curr Opin Pharmacol 18, 2014, pp. 56-60.
4. Lestari ES, Severin JA, & Verbrugh HA. “Antimicrobial Resistance among Pathogenic Bacteria in Southeast Asia.” Southeast Asian J Trop Med Public Health 43(2), 2012, pp. 385-422.
5. Magiorakos AP, et al. “Multidrug-Resistant, Extensively Drug-Resistant and Pandrug-Resistant Bacteria: an International Expert Proposal for Interim Standard Definitions for Acquired Resistance.” Clin Microbiol Infect 18(3), 2012, pp. 268-281.
6. Kostyanev T, et al. “The Innovative Medicines Initiative's New Drugs for Bad Bugs Programme: European Public-Private Partnerships for the Development of New Strategies to Tackle Antibiotic Resistance.” J Antimicrob Chemother 71(2), 2016, pp. 290-295.
7. Fernandes P & Martens E. “Antibiotics in Late Clinical Development.” Biochem Pharmacol 133, 2017, pp. 152-163.
8. Newman MJ. “Food Safety: Take Life Easy; Eat, Drink and Be Merry.” Luke 12: 19b. Ghana Med J 39(2), 2005, pp. 44-45.
9. Fossum S, Crooke E, & Skarstad K. “Organization of Sister Origins and Replisomes during Multifork DNA Replication in Escherichia coli.” EMBO J 26(21), 2007, pp. 4514-4522.
10. Collignon P. “Resistant Escherichia coli - We Are What We Eat.” Clin Infect Dis 49(2), 2009, pp. 202-204.
11. Chudobova D, et al. “Effect of Ampicillin, Streptomycin, Penicillin And Tetracycline on Metal Resistant and Non-resistant Staphylococcus aureus.” Int J Environ Res Public Health 11(3), 2014, pp. 3233-3255.
12. Banerjee R & Johnson JR. “A New Clone Sweeps Clean: the Enigmatic Emergence of Escherichia coli Sequence Type 131.” Antimicrob Agents Chemother 58(9), 2014, pp. 4997-5004.
13. Righi E, et al. “Global Prevalence of Carbapenem Resistance in Neutropenic Patients and Association with Mortality and Carbapenem Use: Systematic Review and Meta-Analysis.” J Antimicrob Chemother 72(3), 2017, pp. 668-677.
14. Keen EC. “Phage Therapy: Concept to Cure.” Front Microbiol 3, 2012, Article 238.
15. Guglielmi G. “Do Bacteriophage Guests Protect Human Health?” Science 358(6366), 2017, pp. 982-983.
16. National Institute of Allergy and Infectious Diseases. NIAID's Antibacterial Resistance Program: Current Status and Future Directions 2014, pp. 1-15.
17. Soni KA & Nannapaneni R. “Bacteriophage Significantly Reduces Listeria monocytogenes on Raw Salmon Fillet Tissue.” J Food Prot 73(1), 2010, pp. 32-38.
18. Soni KA, Nannapaneni R, & Hagens S. “Reduction of Listeria monocytogenes on the Surface of Fresh Channel Catfish Fillets by Bacteriophage Listex P100.” Foodborne Pathog Dis 7(4), 2010, pp. 427-434.
19. Servick K. “U.S. Center Will Fight Infections with Viruses.” Science 360(6395), 2018, pp. 1280-1281.
20. Loc-Carrillo C & Abedon ST. “Pros and Cons of Phage Therapy.” Bacteriophage 1(2), 2011, pp. 111-114.
21. Labrie SJ, Samson JE, & Moineau S. “Bacteriophage Resistance Mechanisms.” Nat Rev Microbiol 8(5), 2010, pp. 317-327.
22. Koonin EV, Makarova KS, & Zhang F. “Diversity, Classification and Evolution of CRISPR-Cas Systems.” Curr Opin Microbiol 37, 2017, pp. 67-78.
23. Mali P, Esvelt KM, & Church GM. “Cas9 as a Versatile Tool for Engineering Biology.” Nat Methods 10(10), 2013, pp. 957-963.
24. Jiang F & Doudna JA. “CRISPR-Cas9 Structures and Mechanisms.” Ann Rev Biophys 46, 2017, pp. 505-529.
25. Nurse P. “Universal Control Mechanism Regulating Onset of M-phase.” Nature 344(6266), 1990, pp. 503-508.
26. Norbury C & Nurse P. “Controls of Cell Proliferation in Yeast and Animals.” Ciba Found Symp 150, 1990, pp. 168-

177; discussion pp. 177-183.
27. Fruhmann G, et al. “Yeast Buddies Helping to Unravel the Complexity of Neurodegenerative Disorders.” Mech Ageing Dev 161(Pt B), 2017, pp. 288-305.
28. Balasubramanian MK, Bi E, & Glotzer M. “Comparative Analysis of Cytokinesis in Budding Yeast, Fission Yeast and Animal Cells.” Curr Biol 14(18), 2004, pp. R806-R818.
29. Brown SD & Lorenz A. “Single-step Marker Switching in Schizosaccharomyces pombe Using a Lithium Acetate Transformation Protocol.” Bio Protocol 6(24), 2016, e2075.
30. Dubin SB, Benedek GB, Bancroft FC, & Freifelder D. “Molecular Weights of Coliphages and Coliphage DNA. II. Measurement of Diffusion Coefficients Using Optical Mixing Spectroscopy, and Measurement of Sedimentation Coefficients.” J Mol Biol 54(3), 1970, pp. 547-556.
31. Mirzaei MK & Maurice CF. “Ménage à trois in the Human Gut: Interactions between Host, Bacteria and Phages.” Nat Rev Microbiol 15(7), 2017, pp. 397-408.
32. Wittebole X, De Roock S, & Opal SM. “A Historical Overview of Bacteriophage Therapy as an Alternative to Antibiotics for the Treatment of Bacterial Pathogens.” Virulence 5(1), 2014, pp. 226-235.
33. Wernicki A, Nowaczek A, & Urban-Chmiel R. “Bacteriophage Therapy to Combat Bacterial Infections in Poultry.” Virol J 14(1), 2017, Article 179.
34. Pires DP, Melo L, Vilas Boas D, Sillankorva S, & Azeredo J. “Phage Therapy as an Alternative or Complementary Strategy to Prevent and Control Biofilm-Related Infections.” Curr Opin Microbiol 39, 2017, pp. 48-56.
35. Huff WE, et al. “Prevention of Escherichia coli Respiratory Infection in Broiler Chickens with Bacteriophage (SPR02).” Poult Sci 81(4), 2002, pp. 437-441.
36. Zimmer M, Scherer S, & Loessner MJ. “Genomic Analysis of Clostridium perfringens Bacteriophage 3626, Which Integrates into Guaa and Possibly Affects Sporulation.” J Bacteriol 184(16), 2002, pp. 4359-4368.
37. Nigam A, Gupta D, & Sharma A. “Treatment of Infectious Disease: Beyond Antibiotics.” Microbiol Res 169(9-10), 2014, pp. 643-651.
38. Sulakvelidze A, Alavidze Z, & Morris JG, Jr. “Bacteriophage Therapy.” Antimicrob Agents Chemother 45(3), 2001, pp. 649-659.
39. Marchant J. “When Antibiotics Turn Toxic.” Nature 555(7697), 2018, pp. 431-433.
40. Chan BK, et al. “Phage Selection Restores Antibiotic Sensitivity in MDR Pseudomonas aeruginosa.” Sci Rep 6, 2016, Article 26717.
41. Bernheim A & Sorek R. “Viruses Cooperate to Defeat Bacteria.” Nature 559(7715), 2018, pp. 482-484.

emerginginvestigators.org