The Existence of Antibiotic Resistant Bacteria in Environmental Sources

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Introduction

The discovery of antibiotics was a turning point in the development of medicine (Blankinship et. al., 2013). Antibiotics were developed to combat disease, but the emergence of antibiotic resistance has dampened their therapeutic use (Blankinship et. al., 2013). Antibiotic resistance is the ability of an organism to reproduce and grow in the presence of a drug that would usually destroy the members of the strain (Merrett, 2013). According to the American Society of Microbiology, an estimated 63,000 people die each year, , from antimicrobial resistant infections acquired in hospitals (Bhandari et. al, 2015).

The initial success seen by antibiotics may be temporary as scientists and medical practitioners expect an ongoing battle with the evolution and existence of antibiotic resistance (Merrett, 2013). Once an antibiotic is widely used, resistant strains increase in frequency (Bhandari et. al, 2015). As a rising threat to global health, drug resistance has the ability to compromise progress made in medicine (Blankinship et. al., 2013).

The potential issue of resistance was identified before the first use of antibiotics. However, it has become a larger threat recently as 70% of known bacteria have developed resistance to at least one antibiotic (Merrett, 2013). Upon the wide use of antibiotics, exponentially increasing numbers of resistant strains emerged. Seven years before the introduction of , sulfonamides, there was already an instance of resistance reported. These

same mechanisms still operate today, almost 70 years later (Merrett, 2013). The introduction of new antibiotics has seen a similar course of events, with increasing use of the drug, resistance appears (Singer et. al, 2016).

Bacteria can transfer genes conferring resistance to one another or acquire it from the environment (Merrett, 2013). The resistance is a threat to the health of the globe and the overuse or misuse of antibiotics and the slow development of new antibiotics only exacerbates the problem. (Williams et. al., 2016). The availability of sub-standard products, occurring most often in low-income nations that can receive medicine without a prescription also contributes to antibiotic resistance (Merrett, 2013). Recently, attention has been given to the existence of bacteria showing resistance in the environment.

Bacteria found in the environment predate the era of modern medicine, and are reported to carry genes encoding resistant to critical antibiotics (Bhandari et. al., 2016). Resistance that is already present in the genome of a bacterium is intrinsic resistance, and is independent of exposure to antibiotics (Bhandari et. al., 2016). Acquired resistance occurs when bacteria are no longer susceptible to antibiotic treatments that they were in the past. These species become resistant after spontaneous mutations in critical genes or acquiring new genes by some transfer from another species. Resistance through intermicrobial transfers often occur through factors like plasmids, which are circular DNA strands in the cytoplasm of a bacterium, being transferred through conjugation, transformation, or transduction (Blankinship et. al., 2013).

Penicillin is an antibiotic that functions by inhibiting the synthesis of the cell wall, which is a layer of peptidoglycan for cell structure and support. This family of antibiotics acts as an irreversible inhibitor of the enzyme transpeptidase, which is needed by bacteria to synthesize the cell wall. Inhibition prevents the final stage of bacterial wall synthesis in

binary fission, and the cell structure is severely weakened and subject to lysis (Chang et. al., 2016). Therefore, penicillin is only active against young, growing cells because they are synthesizing peptidoglycan. This mechanism of action is seen in all penicillin-like antibiotics including ampicillin (Chang et. al., 2016).

Ampicillin is widely used due to its cost-effectiveness, minimal side effects, and relatively high success in treatment (Chang et. al., 2016). The rapid increase of resistance to ampicillin has been seen in both developing and developed countries (Singer et. al., 2016). Understanding the mechanism of resistance of these pathogenic strains can help to design a more effective drug for the future. The most common method of resistance to penicillin-based antibiotics is the alteration of penicillin-binding proteins. Gram-positive bacteria confer this resistance by decreasing the affinity of penicillin to these proteins, which are membrane associated macromolecules that play key roles in cell wall synthesis (Dever and Dermody, 1991). The other mechanism is the production of beta-lactamase, which deactivates the antibiotics by hydrolyzing the amide bond of the B-lactam ring rendering it inactive (Dever and Dermody, 1991). This is found widely across both gram-positive and gram-negative bacteria.

Many experiments focused on antibiotic resistance often study a particular pathogen such as MRSA or are limited to healthcare settings (Blankinship et. al., 2013). However, bacteria in the environment should also be of concern due to the ability of these bacteria to transmit genes. Thus, these bacteria do not need to be exposed to antibiotics or be pathogenic to have the capability to be resistant. Through this experiment, pure bacterial culture isolated from soil will be exposed to antibiotics and their growth assessed to determine the susceptibility to ampicillin. Manipulations in the concentration of antibiotic will be employed to assess if resistance occurred upon initial exposure or gradually acquired.

In tubes increasing concentrations of antibiotics, it is expected to see more resistance upon exposure to the drug. Due to the fast rate of reproduction in bacteria, there are many opportunities for the species to mutate.

The purpose of this study is to understand the prevalence of antibiotic resistance in bacteria isolated from soil, and the capacity a species has to become more resistant upon exposure. Based on previous research, resistance of antibiotics is expected in bacteria obtained from a soil sample.

Methods

Isolate bacteria from soil

A tube was obtained and 5 mL of Nutrient Broth added. Aseptically, with a sterile loop, a small amount of soil obtained from an area around a plant was transferred into the tube. Then the tube was mixed with a vortex machine to suspend any organisms from the soil into the broth. The tube was left to sit at room temperature (25 degrees Celsius) for about 30 minutes to allow soil particles to settle. To streak the plates, only the upper liquid was used to avoid particles settled to the bottom. The T-streak technique was used to streak two NA plates for individual colonies. The plates were put in an incubator at 30 degrees Celsius for 48 hours, and stored at 4 degrees Celsius until use. The original soil extract was also stored at this temperature.

The two plates were examined and difference in colonies noted. Three different colonies were picked to further investigate. Three more NB plates were obtained and streaked again using a single colony from the previous plates. They were incubated at 30 degrees Celsius for 48 hours and stored at 4 until use. Colonies that were abnormal in shape and opaque in color were selected as possible *Bacillus* species.

Enumerate

The colonies of cells were counted on the original plates were counted to enumerate the number of bacteria in the soil sample. It is assumed that each colony resulted form binary fission of one single cell. This gives the colony forming units per volume.

Media to cultivate

Nutrient broth was used in the test tubes as the medium to cultivate growth of bacteria. This is an undefined medium, which makes it richer in nutrients, and the best environment to grow the bacteria.

Method for identifying

In identifying bacteria from the soil, first, a smear was prepared on a microscope slide. A colony from the second set of plates was aseptically transferred to the slide, and a gram stain was performed to determine if the bacteria were gram positive or negative. Through this microscopy process, we were also able determine the shape, dimensions, and arrangement of the cells.

Finally, a sporulating broth was made, and then the bacterium was inoculated into a tube with this broth and put into the incubator for two days. The high salt environment of the broth causes the bacteria to produce spores. In order to determine if spores were being produced, another slide was prepared and the endospore stain performed. After determining the bacterium was gram-positive, rod shaped, forming short chains and in clumps, 1um x .5um in dimensions, and endospore producing, could be confident the bacterium was *Bacillus*.

Antibiotic testing

In testing the ability of resistance of the environmental bacteria, a control was used with no antibiotic ensuring the media provides the bacteria with nutrients to survive without the stressful environment. The first series of tubes a 10-fold serial dilution of the

stock antibiotic solution (50mg/ml) was performed. 4.5 mL of Nutrient Broth was dispensed into 12 tubes (replicates of three). The first dilution received 0.5 mL of a 1mg/ml stock of ampicillin in nutrient broth. This is a 1/10 dilution or 100 ug/ml of ampicillin. 0.5 mL was continually transferred and mixed into the new broth until a 10,000 dilution or 0.1 ug/ml was achieved. Three tubes were used as the control so no ampicillin was present. The tubes were then mixed using the vortex machine. In order to transfer a constant amount of bacteria was inoculated into each tube, the original culture was diluted with nutrient broth to receive an absorbance in the spectrophotometer of 0.100-.105. 10 drops of the culture at this absorbance were pipetted into the new tube. The tubes were then incubated at 35 degrees Celsius for 24 hours After incubation, the tubes were mixed and 1000 ul pipetted into a sterile spectrophotometer cuvette, and absorbance was measured and recorded for every tube. From this range, we can further investigate to pinpoint the concentration necessary to initially kill bacteria that has not previously been exposed to antibiotic.

The second experiment increased the concentration of antibiotic present in each tubes. Three tubes were made at each concentration of 1.6 ug/ml, 8 ug/ml, 40 ug/ml, 200 ug/ml, and 1000 ug/ml. Then all tubes were inoculated with the original culture of *Bacillus* at .100-.105 absorbance, and incubated at 35 degrees Celsius for 24 hours. The tubes were then mixed using the vortex machine, and 1000 ul pipetted into a sterile spectrophotometer cuvette, and absorbance was measured for every tube. The next set of tubes were based on these results in order to refine the range at which all cells are killed.

Tubes were made at concentrations of 400 ug/ml, 500 ug/ml, 600 ug/ml, 700 ug/ml, 800 ug/ml and 900 ug/ml. Each concentration had three tubes, and all were inoculated with the original culture of *Bacillus,* and incubated at 35 degrees Celsius for 24

hours. The tubes were then mixed using the vortex machine, and 1000 ul pipetted into a sterile spectrophotometer cuvette, and absorbance was measured for every tube.

Pushing Resistance of Bacteria

The next experiment was to determine if changes in resistance could be seen upon exposure to antibiotic. Tubes were made with the following concentrations with the same methods as used before: 625, 650, 675, 700, 725, 750, 775, and 800 ug/ml. Bacteria growing in the 600 ug/ml culture was used to inoculate the 625 ug/ml. In order to be sure a constant amount of bacteria was inoculated into each concentration in the future, the culture was diluted with nutrient broth to receive an absorbance in the spectrophotometer of 0.100-.105. 10 drops of the culture at this absorbance were pipetted into the new tube. After 24 hours of incubation, the tubes were subsequently put into the spectrophotometer and their absorbance measured and recorded. The procedure was continued at each concentration, a .100-.105 absorbance of bacteria from 625 ug/ml was used to inoculate the 650, incubated for 24 hours, then read in the spectrophotometer. This process was continued until the spectrophotometer reading was less than the absorbance used to inoculate the culture (.100).

In order to test if the bacteria was evolving under the stressful condition of the antibiotic, the bacteria from the 750 ug/ml tube was used in lower concentrations of antibiotic. Again, an absorbance of .100 was used to have a consistent amount, and then 10 drops were pipetted into tubes at 200, 400, 500, 600, and 700 ug/ml. The tubes were incubated at 35 degrees Celsius for 24 hours. After incubation, the absorbance in each tube was tested.

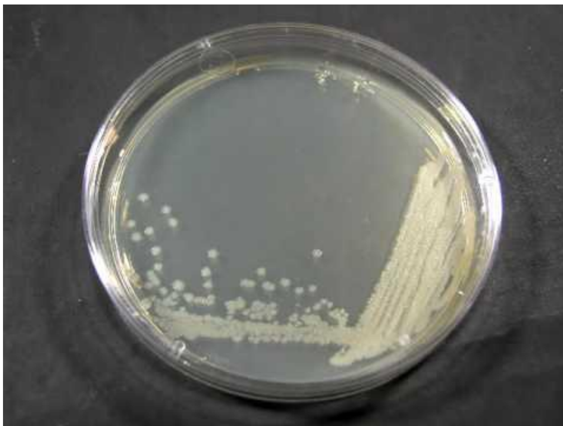
Gradient Plates

Finally, a group of plates were made in order to create a concentration gradient of antibiotic across the plate. Nutrient agar was prepared and autoclaved. Ampicillin was added to achieve 750 ug/ml. The plate would range from 0-750ug/ml. One colony from control plates of the original and pushed cultures were poked with a toothpick and placed into one ml of nutrient broth. These tubes were then incubated for two hours, and then 150 ul from this culture was pipetted onto the gradient plates and spread using the spread plate method. The plates were incubated for 8 hours, and after incubation colonies present counted.

Results

Method for Identifying

The colony selected was white and dull in color with irregular shape. The colonies were raised off the surface. A gram stain revealed the bacteria were gram-positive. This also showed the bacteria was rod-shaped and grew in chains. The size of an individual bacterium was 0.5 um by 1.0 um. Next, an endospore stain indicated that the bacteria also grew endospores. Based on these results, and the appearance of the colony on the plate, it was concluded that the bacteria species was *Bacillus.*



Antibiotic Testing

In order to determine the upper end of the bacteria's ability to be resistance, first a ten-fold serial dilution was performed. Resistance to the antibiotic was prevalent in each dilution. A regression analysis was performed assessing the relationship, and found a highly significant inverse relationship between concentration of antibiotic and absorbance ($r^2 = 0.60$, df = 10, $p = 0.003$).

Next, based on findings in the serial dilution, the concentration of antibiotic was increased in order to find the upper limit. The upper limit of the bacteria from the original culture from the soil was found to be 600 ug/ml. A regression analysis was performed, and again a significant inverse relationship between concentration of antibiotic and absorbance was present ($r^2 = 0.89$, df = 13, $p < 0.001$). As concentration of antibiotic increases, the absorbance and growth of the sample decreases.

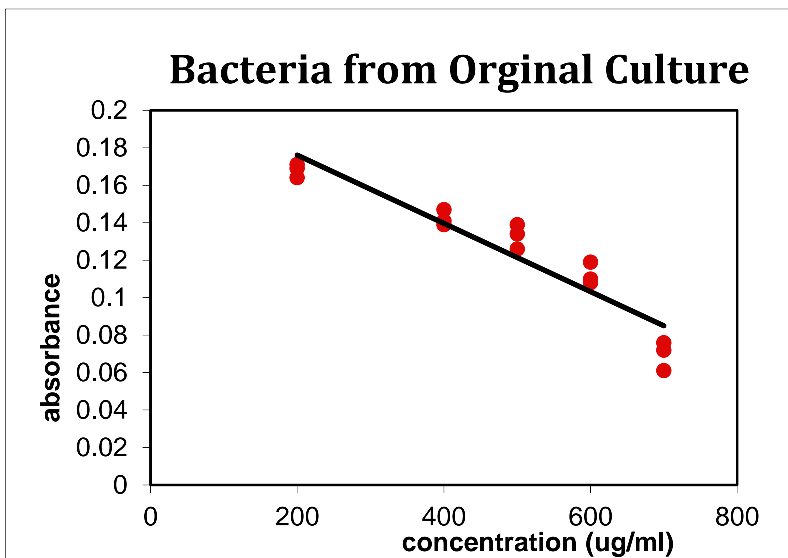


Figure 1: Original culture from soil used to inoculate tubes with increasing concentration of antibiotic and placed in spectrophotometer to assess growth (through absorbance) ($r^2 = 0.89$, df = 13, $p < 0.001$).

Pushing Resistance of Bacteria

The ability to be resistance to higher values of antibiotic after subsequent exposure to antibiotic was tested next. Resistance was able to be pushed upon previous exposure to antibiotic. A regression analysis was used to assess the relationship between absorbance

and concentration of antibiotic was again found to be highly significant ($r^2 = 0.91$, df = 16 $p < 0.001$).

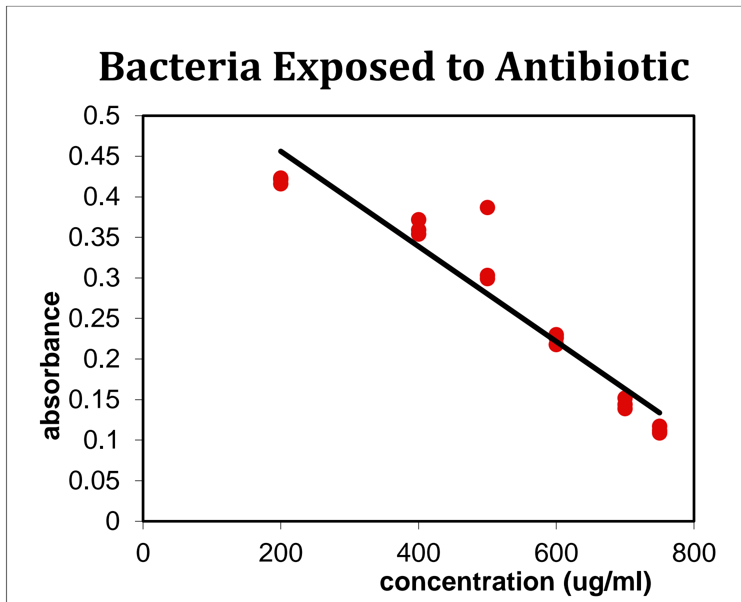


Figure 2: Bacteria taken from growth in highest exposed level of antibiotic used to inoculate tubes of increasing concentration and placed in spectrophotometer to assess growth (through absorbance) ($r^2 = 0.91$, df = 16 $p < 0.001$).

The concentration of antibiotic was pushed in order to observe the point at which the cells were no longer able to grow even upon exposure. Absorbance readings began to be under .100 at 775 ug/ml, therefore, resistance could be pushed up to 750 ug/ml.

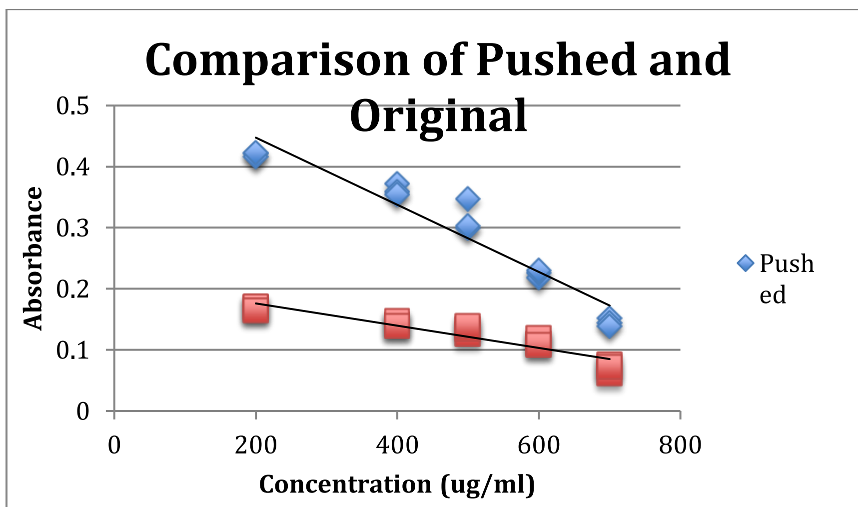


Figure 3 : Depicts the growth of the original culture and pushed culture (F = 132.38, df = 28, $p < 0.001$).

Finally, the bacteria exposed to high levels of antibiotic, was grown lower levels of antibiotic to compare growth to the original culture. In all instances, the growth in the tube exposed to antibiotic had a higher absorbance reading at each concentration, and therefore higher growth. An Analysis of Covariance was used since one variable can be held under statistical control (concentration) while comparing the difference in the other (absorbance). The results determined the absorbance of the original and pushed were significantly difference ($p < 0.001$).

Gradient Plates

Gradient plates were used to visually quantitate that the pushed culture has changed in some way to better survive than the original culture. From the number of colonies and spread across the plates, it was concluded that the pushed culture had changed. The pushed culture plates contained an average of 71.33 colonies, while the original had an average of 30.33 colonies present. These plates showed the pushed culture had a greater ability to grow.

Discussion

In the study bacteria was extracted from a soil sample and exposed to varying levels of antibiotics to determine the intrinsic resistance. Previous research described the ability of environmental bacteria to be resistant to physiological doses of antibiotic, but the extent to which and ability to push was not well recorded. In testing the ability of the bacterium, it was found that resistance to the antibiotic highly exceeded the physiological dosage ranging from 3.0- 4.3 ug/ml. The bacteria showed capabilities of being resistant up to 600 ug/ml. This environmental bacterium had the capability to be resistant to high levels of antibiotic upon initial exposure (Fig 1). However, growth conditions in a nutrient rich broth medium are much different from growth in the human body, which might explain the

greater resistance. Based on this data, this bacterium had a gene or sets of genes necessary to live in an environment that should have caused it to die, therefore showing intrinsic resistance.

Bacteria are extremely versatile and adaptable, and their rapid rates of cell division allow more changes to occur in the genome. This bacterium had the ability to become more resistant after exposure to high levels of antibiotic (Fig 2). Along with being intrinsically resistant, the bacterium was able to become increasingly resistant through gene mutation or transfer of genetic material. Although advantageous mutations are very rare, bacteria multiply and divide so quickly they have a higher chance of a mutation occurring that will help them to survive. This bacterium could have also achieved new genetic material from another containing the genes conferring resistance. Yet another way this bacterium could have become increasingly resistant is through phenotypic plasticity. This occurs when one genotype has to ability to produce more than one phenotype when exposed to different environments.

After becoming more resistant upon exposure, the bacteria were able to grow and survive better in lower doses of antibiotic. When put in lower levels of antibiotic, the bacteria previously exposed grew significantly more than the original culture that had not been previously exposed (Fig 3). Not only are the bacteria conferring resistance when exposed in harsh environments, but also the resistance is becoming a part of the genome and showing a greater ability to survive and reproduce under less stress.

The gradient plates were another mechanism used to show how the bacteria developed or acquired some advantageous gene allowing them to survive better and growth more. Colonies were more numerous and spread more evenly over the plate with the bacterial culture that was being maintained in broth with antibiotic. However, the

colonies were less and confined to the side of the plate with lower concentrations of antibiotic with the bacterial culture that had never been exposed. Although it shows differences in ability to survive and grow, initial resistance is still present.

Recent research has highlighted soil and water environments as recipients, reservoirs, and sources of antibiotic resistance genes (Warren, 2016). Both of those environments have high human interaction, therefore, creating an opportunity for the bacteria to then come into contact with people. Commonly interacted with surfaces such as cell phones or public areas such as computer keyboards and door handles will serve as prime candidates for the transmission of antibiotic resistance genes from person to person (Blankinship et. al, 2013). Surveillance on the organisms present on these surfaces in the future can provide crucial information to scientists and medical professionals. This information can then be used in order to develop strategies against the spread of antibiotic resistance.

There are other antibiotics being tested to debunk the ability of super bugs to render modern medicine inactive, but testing can take years before it is on the market (Warren, 2016). As antibiotics continue to be overprescribed and no new developments occurring, the existence of antibiotic resistance is expected to increase (Warren, 2016). The World Health Organization (WHO) and its Global Action Plan has outlined strategies to tackle antimicrobial resistance (AMR) (Singer et. al, 2016). These five strategies include improving awareness and understanding of AMR, strengthening knowledge through research, reducing incidence of infection, optimize the use of antimicrobial agents, and guarantee sustainable investment in countering AMR (Singer et. al, 2016).

Works Cited

Bhandari, D., Thapa, P., Thapa, K., Tandukar, S., and Sherchand, J. B. Antibiotic Resistance: Evolution and its Alternatives. (2015). Canadian Journal of Infection Control. Vol. 31. No. 3. P. 149-155.

Blankinship, L. A., Cotton, B. L., and Gaston, J. L. Survey of Antibiotic Resistance in Cell Phone and Computer Keyboard Isolated Bacteria. (2016) University of North Alabama Biology Department. Vol. 84. No. 3. P 165- 172.

Chang, C., Lin, H., Li, B., and Li, Y. A Novel Metallo-B-Lactamase Involved in the Ampicillin Resistance of *Streptococcus pneumonia* ATCC 49136 Strain. (2016). Department of Applied Chemistry. Vol. 11. No. 5. P 1-13.

Dever, LA. and Dermody TS. Mechanisms of Bacterial Resistance to Antibiotics. (1991) Arch Internal Med. Vol. 151. No. 5. P 886-895.

Merrett, G. L. Tackling Antibiotic Resistance for Greater Global Health Security. (2013) Centre on Global Health Security. P 1-16.

Singer, A. C., Shaw, H., Rhodes, V., & Hart, A. Review of Antimicrobial Resistance in the Environment and Its Relevance to Environmental Regulators. (2016). *Frontiers in Microbiology*, *7*, 1728.

Warren, E. (2016). Antibiotic resistance: what can we do?. *Practice Nurse*, *46*(1), 18-21.

Williams, M.R., Stedtfeld, R. D., Gue, X., and Hashsham, S. A. Antimicrobial Resistance in the Environment. (2016). Water Environment Research. Vol. 88. No. 10. P 1951- 1967.