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The Road to an Improved Magic Bullet: Combating Antibiotic Resistance in the Genetic Era

Jibin J. Abraham
Regis University

THE ROAD TO AN IMPROVED MAGIC BULLET: COMBATING ANTIBIOTIC RESISTANCE IN THE GENETIC ERA

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by

Jibin J. Abraham

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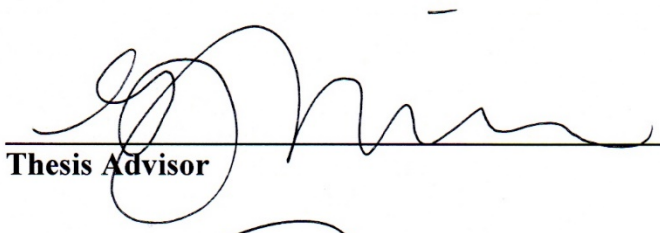
Thesis written by

Jibin J. Abraham

Approved by



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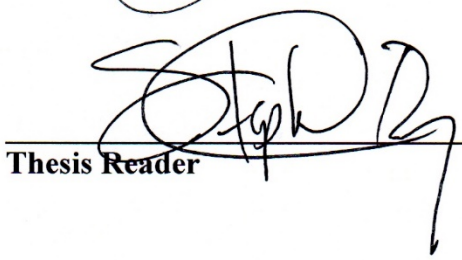

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Introduction

Humans have used antibiotics far longer than most may realize. The oldest known evidence for the presence of antibiotics in humans has been dated back to approximately C.E. 350 – 500 where tetracycline was found in human bones (Nelson et al., 2010). Through the millennia, earlier civilizations applied herbaceous poultices, ate strange soups, or pressed soils and molds into wounds to treat bacterial infections. At the time the people believed these remedies forced out evil spirits and or appeased gods of disease, and were not aware that such illnesses were bacterial in nature. These early treatments were sometimes successful because the antibiotics naturally produced by bacteria found in plants, clay, and soil were likely enough to kill the infectious bacteria which would cure the wound or disease, acting as a rudimentary chemotherapy.

Since the discovery of antibiotics in the 1930s and the clinical application of antibiotics in the 1940s, mortality rates from bacterial infections have been significantly reduced where there is access to antibiotics. However, the widespread use of antibiotics has put substantial evolutionary pressure on bacteria which results in a rapid selection for microorganisms resistant to these treatments. In less than 100 years after the discovery and clinical application of the first antibiotic, antibiotic resistance threatens to outpace the current management of infections. This makes antibiotic resistance one of the most significant global human health threats, with bacteria developing resistance to the current available classes of antibiotics faster than new ones are discovered or synthesized. Medical procedures including diabetes management, organ transplants, and cesarean

sections (C-sections) become significantly riskier if post-operative care is not effective in minimizing infections wherever possible.

A critical factor contributing to the unnecessary use of antibiotics is the lack of rapid and accurate diagnostic tests that can identify the nature of an infectious agent and anticipate pathogenic sensitivities to various drug classes. Successfully addressing this issue will facilitate a shift in antibiotic discovery and synthesis towards more narrow-spectrum, pathogen-specific antibiotics to slow the evolution of resistant bacteria, as well as promote antimicrobial stewardship. Current methods for addressing antibiotic resistance are too slow and ineffective. Recent efforts have called attention to the need for a new approach to detection and treatment, and there exists a potential technology that is fast, specific, controllable, and cheap. This will impact our current approaches to antimicrobial stewardship, which the Society for Healthcare Epidemiology of America (SHEA) defines as “coordinated strategies to improve the use of antimicrobial medications with the goal of enhancing patient health outcomes, reducing resistance to antibiotics and decreasing unnecessary cost” (Society for Healthcare Epidemiology of America, 2017).

At the precipice of a new era in healthcare in the developed world, one where most decisions may soon be personalized to a patient’s genetics and background, the potential for disruption in the medical field is significant. The development of CRISPR technology has much excitement and hype around its possible utilization, and rightly so, as it may hold the key to placing a new era of robust, long-lasting treatments in our reach, including applications to resolve previously untreatable conditions and fatal infections.

There are many articles that report on this as though this may be the end-all-be-all of medical treatment in the future. However, the rate at which this is developing may be too quick for key discussions regarding its future use, and as such may lead to hasty conclusions and decisions, delaying and possibly hindering its implementation and making the process much more challenging than it needs to be. Considering the current application of the technology was first identified one decade ago in 2008, there is still much to research before integrating it as a replacement for other working methods. It may just be fitting, however, that a rapidly-developing field may be able to address a global issue rapidly outpacing our ability to fight it: antibiotic resistance. First, by using CRISPR technology in detection and diagnosis outside the body, then possibly to supplement or enhance treatment within the body, and, perhaps in another decade or so, as a replacement for antibiotics altogether.

To explore this topic, the thesis will begin with an introduction to microbiology to place this conversation within the context of physical scale. To establish the urgency of antibiotic resistance, the second chapter is dedicated to describing the problem, its history, current approaches, and why more needs to be done. The second chapter will also introduce Paul Erhlich who coined the term “magic bullet” as an idealistic gold standard and whose work had profound effects on the state of healthcare and medical science in the decades since his time. CRISPR, the new technology that places us in a new era in medicine, is covered in the third chapter. Finally, the fourth chapter will briefly propose general suggestions surrounding the approach to the problem of antibiotic resistance as to be addressed by CRISPR.

1. Introduction to Microbiology

1a. Brief history of microbiology.

The discovery of bacteria is credited to Anton van Leeuwenhoek (1632 – 1723), who is considered the father of the field of microbiology. By trade, van Leeuwenhoek was a Dutch textile merchant who did not receive a formal education. His work required him to inspect weaves and fibers of textile products from different manufacturers, and microscopes provided the best way to inspect the materials. Anton van Leeuwenhoek became very much intrigued by the manufacture of glass and its various intrinsic properties. In 1671, he developed a microscope powerful enough to view bacteria using only sunlight to backlight the subject. He observed what he called 'animalcules,' which are now known as bacteria. Van Leeuwenhoek was the first to observe bacteria, and by his death in 1723 he had invented more than 400 microscopes. His contributions developed and established the field of microbiology, which is the study of microorganisms.

While various scientific societies and researchers began to describe bacteria and its various forms, many were unsure of how bacteria formed. In September of 1655, an English natural historian named Robert Hooke (1635 – 1703) published *Micrographia*, which was the first book describing microscopic observations. One of Hooke's most famous observations was of two pieces of thinly-sliced cork, in which he was able to see rigid box-like structures comprising the entire cork. These structures Hooke had observed

are now known to be the cell walls of the cork bark, and Hooke called these structures ‘cells’ because they resembled the sleeping quarters of monks, which the monasteries often referred to as cells.

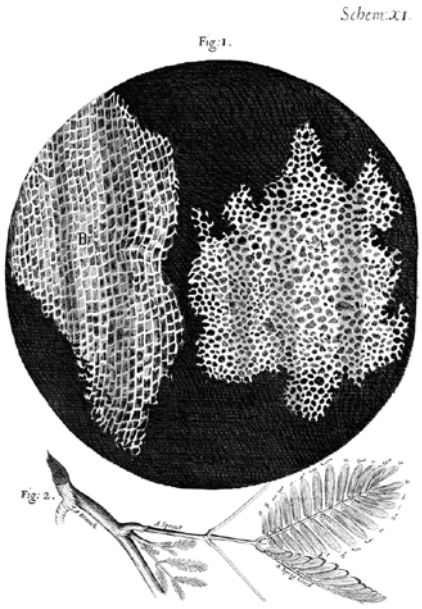

Figure 1. Robert Hooke’s sketches of two thinly-sliced pieces of cork bark at 50x magnification in which he viewed empty space enclosed by box-like frames, and termed these ‘cells’ (Hooke, 1665).

1b. Koch’s postulates.

While in ancient civilizations the causes of infections were unknown, it is now known that the majority of infections are bacterial or viral in nature. The invention of van Leeuwenhoek’s microscope allowed for research on microorganisms which had not been observed before 1671. As soon as microscopes were more widely distributed, it was

theorized that bacteria could be responsible for various infections, and it seemed clear that specific species or forms were causative agents for different infectious diseases (pathogens). Indirect evidence of microorganism pathogenicity was provided by the widespread adoption and implementation of sterile technique in surgery and the washing of hands in hospital settings which reduced the number of infections and post-operational recovery time. However, there was not enough evidence to definitively prove that bacteria caused disease.

Robert Koch (1843 – 1910) was a German physician. He is called the father of medical microbiology for having developed and defined the concept of infectious disease and for providing convincing evidence. Koch's early work studied anthrax which is a disease that generally affects cows, but can also affect humans. He was able to isolate a bacterium known as *Bacillus anthracis* from the blood of cows showing symptoms of anthrax, which demonstrated that bacteria were present in the blood of infected individuals. However, this was only the association of *B. anthracis* with the symptoms of disease and not proof that the bacterium is the causative agent, or pathogen. In 1877, he developed a framework in which one could confirm whether a bacterium was the pathogen in an infection. The four postulates are as follows:

1. Isolate the pathogen from infected individuals. The suspected pathogen must be present in all infected individuals and absent from healthy individuals.
2. The suspected pathogen must be developed in a pure laboratory culture.
3. Cells from the pure laboratory culture must then be injected into a healthy, uninfected test subject(s) (usually a mouse) which must then develop an infection and present the symptoms or die.

4. The suspected pathogen must be reisolated from the organism(s) infected in postulate 3 and shown to be the same pathogen as the original isolated from postulate 1.

When studying anthrax, Koch had shown that bacteria was present in the blood of infected organisms (postulate 1). He realized that the bacterium could be grown outside of a host in a fluid containing all necessary nutrients and retain the ability to cause disease when injected into a healthy organism (postulates 2 & 3). Koch injected a small volume of blood containing the bacterium into a healthy, uninfected mouse which developed anthrax soon after (postulate 3). He drew some blood from the newly-infected mouse and injected that blood into a second healthy, uninfected mouse which then also showed the characteristic symptoms (postulate 3). However, Koch knew that bacteria can exhibit different forms (morphologies) and look different in a lab culture, and may express properties that can present as different symptoms in an infection. Koch added the 4^{th} postulate to minimize any possibility of confusion or misattributing disease to the wrong pathogen. With Koch's postulates, it was finally possible to demonstrate that bacteria caused diseases, and various infectious agents were soon identified and associated with their respective infections.

Why does this matter? These postulates form the basis of identifying pathogens and diagnosing infectious diseases. Of course, the exact method employed by Koch would not be ideal in diagnosing general infections today as it would require significant resources, a large number of rats, and time that may not be available to an infected individual. However, this is still a method by which infectious agents are identified. In

the time since Koch, there are new ways to identify pathogens, which will be covered later in this thesis.

2. Antibiotic Resistance

2a. History of antibiotics.

Prior to the discovery of bacteria, the identification of bacteria as infectious agents, and the discovery of antibiotics, people desperately sought treatments and cures for their illnesses, believing and theorizing various causes of disease. As a result, bizarre treatments have been recorded throughout history, such as using leeches to remove 'bad' blood, following elaborate rituals to appease gods and spirits, applying various pastes and powders to wounds, drinking questionable tonics, and even smoking or injecting crystal methamphetamine, among other suspicious prescriptions.

Several ancient treatments have demonstrated some efficacy against infectious disease, and have been discussed for clinical application in recent years. However, this is because the successful treatments delivered some form of antibiotic, though it was not known at the time. For example, the Egyptians applied a poultice of moldy bread to infected wounds, in which the mold produced antibiotics and killed the infectious bacteria, effectively reducing the period of illness and possibly preventing death. In 1971, Chinese researchers at the Institute of Traditional Medicine in Beijing looked at a 4th century CE text and discovered a method to extract artemisinin from *Artemisia* plants (used in Chinese medicine for millennia) that proved to be an effective treatment against drug-resistant malaria and is now used around the world (Hsu, 2006). Another recent study found a nearly 1,000-year-old recipe for an eye salve of garlic and onion that was

comparably effective to the current treatment of choice for killing a modern superbug (Harrison et al., 2015). The same manuscript, however, also contained salves against elves, and recommended whipping lunatics with a whip made of porpoise skin as a cure. Evidently the manuscript writer did not truly know what was effective or how the supposed cures worked, but the mechanisms for treatment can be understood and identified today. However, to understand how these can studied, it is perhaps pertinent to understand some of the context and work that made such an advancement possible.

2a. i. Paul Ehrlich and the magic bullet.

Some individuals actively sought better treatment options, looking for ways to treat the population at large and minimize infection. Through Koch's work, bacteria were shown to be a source of infection, and researchers set their sights on developing diagnostics and treatments for bacterial infections. German Jewish physician Paul Ehrlich (1854 – 1915) was one such individual, notable for his contributions to early diagnostic and pharmaceutical science, oncology, for fathering the concept of chemotherapy, among other things. Perhaps his most prestigious accolade is his Nobel Prize in Physiology or Medicine which he received in 1908 for his molecular side-chain theory of immunity. Ehrlich's career is worth briefly examining because he was poised to set the stage for drug development in a period that would see the rise of the chemical industry, and recent advances have set a similar stage for the rise of the genetic industry, of which one recent advancement will be discussed later in this paper.

At the start of his research career in the 1870s, Paul Ehrlich developed a personal interest in the use of dyes that could stain tissue. His older cousin Carl Weigert (1845 – 1904) developed the use of dyes like aniline to stain anatomical tissue and preferential staining of bacteria, so Erlich's interest was certainly an inspired one (Jay, 2001). Pursuing this, he developed and published a doctoral thesis about his methods for staining different types of cells—a process during which he discovered mast cells which are important components of the normal immune system (Crivellato, Beltrami, Mallardi, & Ribatti, 2003). He became a physician at the Charité in Berlin, Europe's largest university hospital, where the head of the clinic was Friedrich Theodor von Frerichs (1819 – 1885), who was a professor of internal medicine well-recognized for his contributions surrounding liver and kidney diseases. Von Frerichs was interested in incorporating laboratory diagnostics into clinical treatment and was very accommodating of Ehrlich's staining experiments in animals (Travis, 1989). Through several years of staining tissue samples from patients, Ehrlich became familiar with various dyes intended to show different structures, and was even able to differentiate between bacterial infections based on stains which allowed him to provide diagnostic advice for treatment.

Ehrlich was greatly inspired by Koch's research and the new concepts surrounding infection. When he worked with Koch in the early 1880s, he used his acquired knowledge of dyes to improve several of the staining techniques Koch used and demonstrated his advanced ability to differentiate bacteria in this manner (Thorburn, 1983). Realizing the potential for further application, Ehrlich expressed interest in developing therapeutic uses for the dyes. Koch offered Ehrlich lab space and assistants in

1890, and Ehrlich pushed his animal experimentation. Ehrlich observed something that year: one of the dyes, methylene blue, not only stained the tuberculosis bacteria in the mice he was feeding the dye to, but it also deposited in the axons of the nerve cells of mice without dyeing anything else (Winau, Westphal, & Winau, 1996). The realization came that some dyes exclusively stained bacteria while others also affected a test subject's own cells. This inspired Ehrlich to reason that there may be chemical compounds he could use to selectively stain bacteria within an individual, and perhaps could selectively kill infectious agents without harming the human body. He would eventually come to call this idea the "magic bullet."

The magic bullet concept was the idea of injecting a chemical agent into the blood to specifically target and kill infectious pathogens without harming any human cells. For 15 years he continued to work with mice and tested various dyes, poisons, and toxins to find a way to accomplish a therapy for treating infections. In 1896, Ehrlich founded his own institute, The Institute for Serum Research and Serum Testing, in Steglitz, Germany, and it moved to Frankfurt, Germany three years later where it was renamed The Royal Prussian Institute for Experimental Therapy (Bäumler, 60-61). He turned his focus to trypanosomes in 1901 after reading papers wherein it was revealed these microorganisms caused sleeping sickness. Trypanosomes are a parasitic protozoan, which are not bacteria, but are large microorganisms which made them ideal for evaluating stain effectiveness because they are easier to see. Ehrlich and his assistant, Dr. Kiyoshi Shiga (1871 – 1957) after whom the bacterial genus *Shigella* is named, tested several hundred arsenic-based compounds. Initially, results were less than encouraging because the dyes would stain

both the bacterial cells and the cells of the mice they were testing on. In 1903, after testing more than 500 compounds, Ehrlich found a substance that killed trypanosomes and effectively cured the mice of sleeping sickness (Ehrlich & Shiga, 1904; Felsenfeld, 1957). He called this trypan red. This victory was short-lived, however, as it became rapidly evident that the compound could not kill all trypanosomes, and soon the trypanosomes developed a sort of tolerance to the trypan red, in which case the effective dosage was no longer enough to kill the trypanosomes. However, this was enough to excite Ehrlich and he began to pursue this further in the hopes of finding a real magic bullet.

In 1904, Ehrlich turned his focus to syphilis which is caused by the bacterium *Treponema pallidum*. Syphilis is sexually-transmitted disease in which the first and second stages present as sores and rashes, and can ultimately result in neuronal diseases and infection of the brain. Ehrlich was unsatisfied with the standard treatment of syphilis at the time which utilized inorganic mercury compounds that were mildly effective at best, but was also delivered in toxic concentrations and caused undesirable side effects (Tampa, Sarbu, Matei, Benea, & Georgescu, 2014). In 1907, several papers discussed the use of an arsenic-derived compound called atoxyl that showed some effectiveness in treating sleeping sickness (Boyce, 1907; Breinl & Todd, 1907). Ehrlich, with German chemist Alfred Bertheim (1879 – 1914) and Japanese bacteriologist Sahachirō Hata (1873 – 1938), tested modified versions of atoxyl in syphilis-infected rabbits. For 2 years there was no sign of success, until in 1909 when they tested the 606th compound. Compound 606 was named arsphenamine, and was sold to consumers as Salvarsan by

Hoescht AG, a German life sciences company (Ehrlich & Hata, 1909). This became the first effective treatment for syphilis with few adverse side effects, and was one of the most prescribed drugs for the next 30 years—the first magic bullet.

Paul Ehrlich's career as discussed here represents decades of research and the dedication to searching through hundreds of potential treatment options. Through his persistence, Ehrlich achieved his goal of finding a magic bullet treatment that, when injected into the blood, was selectively absorbed by infectious bacteria without causing damage to human cells. This need for selectively is still required today, but the modern threat of antibiotic resistance has placed significant pressure on researchers to find new and effective treatments, and may soon outpace the progress of research in this field. Ehrlich set the standard of pharmaceutical development with regards to various drugs. Despite the brilliance of Ehrlich's methodical approach to research, however, the approach took far too much time. Screening the effectiveness of individual chemical compounds for selective microbial killing activity is a process that has been streamlined and made much more efficient with new knowledge surrounding the targeting of bacterial cell structures. This is all possible due to a famously "lucky" accidental discovery.

2a. ii. Alexander Fleming, Howard Florey, Ernest Chain, and the discovery of penicillin.

Many individuals are familiar with the discovery of penicillin. In 1928, Scottish physician and microbiologist Sir Alexander Fleming, FRS, FRSE, FRCS (1881 – 1955)

was conducting research on various *Staphylococcus* species—a bacterial genus known to cause painful skin rashes, boils, nausea, and vomiting—and had to leave the plates exposed to air. On September 3rd after returning from a vacation, Fleming began sorting through the plates of *Staphylococcus* colonies and discovered a large mold growing on one of the plates. Mold itself is not unusual when plates are left exposed to air. However, what interested Fleming was that around the mold was a clear 'halo' wherein bacteria would not grow within the halo and those that did were largely clear as a result of lysis, or cell killing (Fleming, 1929).

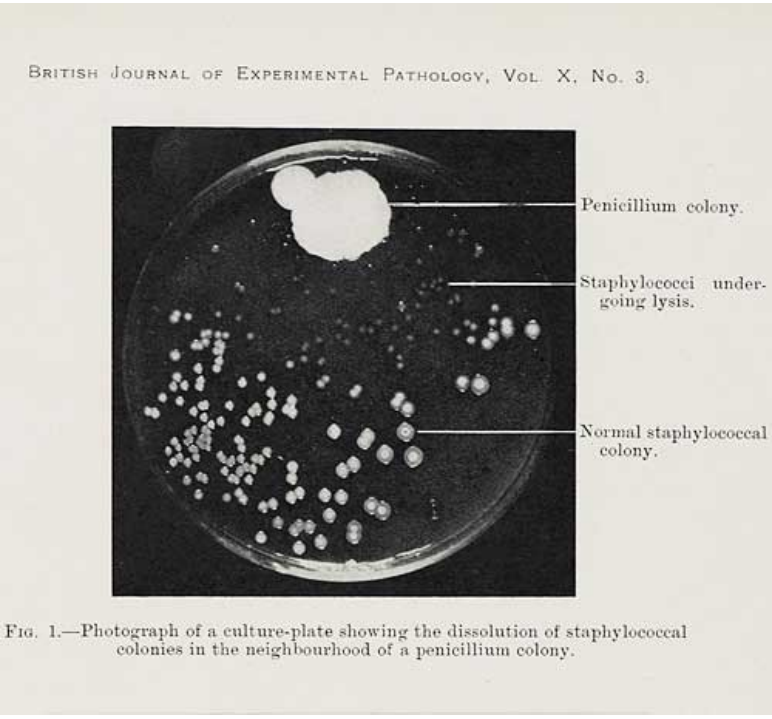

Figure 2. Alexander Fleming's photograph of a bacterial plate growing *Staphylococci* colonies and a *Penicillium* colony exhibit a clear area (zone of inhibition) around the *Penicillium* mold (Fleming, 1929).

As visible in Figure 2, the halo around the *Penicillium* mold was a result of something preventing the bacteria from growing around it. This indicated to Fleming that the mold was secreting something that could kill *Staphylococcus* species. He isolated this chemical and called it penicillin, and tested the compound on 7 common infectious species at once, showing that the chemical was effective against multiple bacterial types (Fleming, 1929). However, Fleming did not believe there was significant potential for treating infections, instead emphasizing its potential in the laboratory for isolating bacteria resistant to the *Penicillium* fungus. He tasked his research assistants with the purification of penicillin, but ultimately only a crude product could be isolated, and Fleming's 1929 paper suggests possible therapeutic uses without any sense of urgency or excitement. He was still interested in the purification of penicillin and provided the specific *Penicillium* strain to anyone who asked for the next 12 years.

Fleming eventually abandoned hope that penicillin could be isolated, but in 1940 an Oxford University pathologist, Sir Howard Florey, OM, FRS, FRCP (1898 – 1968), and an Oxford University biochemist, Sir Ernest Chain, FRS (1906 – 1979), were able to isolate and mass produce the chemical. The two were attempting to discover various structural and mechanistic properties of compounds that demonstrated antibacterial activity, and among the compounds they decided to test was penicillin. To do this, they required large amounts of these antibacterial chemicals, including penicillin, for testing in mice, so they approached various British drug manufacturers, but the economic instability of World War II made corporations weary of taking many risks. The pair devised a method that allowed them to isolate the penicillin, though the process was

inefficient considering 500 liters of mold filtrate was needed to produce the amount of penicillin needed to inject 4 mice (Chain et al., 2005).

Recall that Paul Ehrlich had actively sought a chemical of some sort that could selectively kill bacteria without harming the host—a magic bullet. Like Ehrlich, Florey and Chain relied on animal testing, but utilized a different approach. Ehrlich's method was to infect a mouse and then attempt to dye and kill bacteria without harming the cells at whatever dosage he was using, whereas Florey and Chain first injected penicillin to ensure there would be no toxicity. Fleming had earlier tested a small dose of penicillin in mice to confirm against toxicity, but Florey and Chain injected a larger dose which also showed no negative side effects. In the second part of the experiment, the Oxford pair delivered a fatal concentration of *Streptococcus* bacteria to 8 mice, and injected penicillin into 4 of the infected mice. Less than 17 hours later, all infected mice without penicillin died, as expected, but those with injected penicillin survived and recovered.

This critical 1940 experiment essentially confirmed the antibacterial behavior of penicillin without harming the host, and the significance of Fleming's discovery was suddenly understood. It was first used to treat a patient the following year, and though the patient began to make a recovery, there was not enough penicillin to kill all the bacteria and he died several days later. Manufacturing the drug at a large scale proved to be a challenge, so the Oxford pair traveled to the United States in July of 1941 under the sponsorship of the Rockefeller Institution to find a manufacturer for penicillin because the United States was less involved in war efforts and were less constrained than English companies (Denton, 2013). In 1942, after several companies began production of

penicillin, 11 American patients with various bacterial infections were successfully treated. It seemed that another one of Ehrlich's magic bullets had been found.

The benefits of penicillin in the battlefield became immediately obvious to the U.S. War Protection Board who subsequently created a penicillin program involving 21 companies in 1943 (Bud, 50). Suddenly, all priority was on the mass production of penicillin which was then used to treat a majority of war infections from 1943 – 1945, and this is sometimes credited with having tipped the scales against Adolf Hitler and his forces because fewer Allied men were lost and could rejoin the battlefront. Fleming, Florey, and Chain shared the 1945 Nobel Prize in Physiology or Medicine for the discovery and development of penicillin. The drug became available to the general commercial market and had saturated local drugstores by mid-1945. This marks the beginning of the first widespread antibiotic.

2b. Introduction to antibiotics and modes of action.

Antibiotics are a variety of biomolecules produced by microorganisms that inhibit the growth of other microorganisms or kill them. Though new antibiotics are readily found in nature, less than 1% can be further developed for clinical application in humans. Antibiotic drugs either kill bacteria directly (bactericidal) or inhibit the growth of bacteria (bacteriostatic) which will then be destroyed by host immune defenses or excreted. When categorizing their effective range of bactericidal or bacteriostatic ability, there are two major types of antibiotics; broad-spectrum antibiotics, and narrow-spectrum antibiotics.

Broad-spectrum antibiotics can be active against several types or families of bacteria, whereas narrow-spectrum usually have limited activity and can only be used to treat one or a few bacteria species. The majority of antibiotics used in treatment are produced by the *Streptomyces* genus, which are type of soil bacteria (de Lima Procópio, da Silva, Martins, Azevedo, & de Araújo, 2012). The *Penicillium* mold, which is a type of fungus, inhabits a number of surfaces, including various soil environments. Soil teems with many small life forms and, as a result, there is much competition for access to nutrients and room to grow. Bacteria evolved antibiotics as mechanisms of competition. As previously discussed, the benefit of using antibiotics were demonstrated by Florey and Chain in 1940. Researchers came to realize that the antibiotics were a way of inhibiting the growth of bacteria and began examining the relationships between various soil bacteria to find new antibiotics to use. Upon initial release, penicillin was a very strong antibiotic and used widely, but more antibiotics have since been discovered and are used to clinically different types of bacterial infections.

There are several mechanisms by which various types of antibiotics work, and antibiotics are generally classified by the bacterial structures they target. Unlike Paul Ehrlich's quest to find a chemical that completely and lethally stains bacteria, antibiotics target various biological structures and metabolic or genetic pathways. While there are many classes, the majority of antibiotics inhibit cell wall synthesis, DNA synthesis, or protein synthesis (Walsh, 2003).

2b. i. Cell wall synthesis inhibition | β-lactam antibiotics

Antibiotics that inhibit cell wall synthesis fall largely under the category of β-lactam antibiotics, the majority of which are penicillin derivatives and cephalosporin derivatives. β-lactam antibiotics are generally bactericidal, meaning they kill bacteria because they disrupt the final stage of bacterial cell walls, which are made of peptidoglycan—a type of macromolecule made from amino sugars and short peptides. With a weakened cell wall, or without a closed cell wall, a bacterial cell will burst and become exposed to its surroundings, meaning it cannot regulate its internal environment, and as a result the cell will die. This is what was occurring on Alexander Fleming's plate (Fig. 2). These antibiotics do not negatively affect humans because human cells have plasma membranes made of glycolipids (carbohydrates and lipids), and lack peptidoglycan structures.

The class name β-lactam antibiotics comes from a core structure of a four-membered ring with an amide, as highlighted in the structure of penicillin shown on the following page (Figure 3).

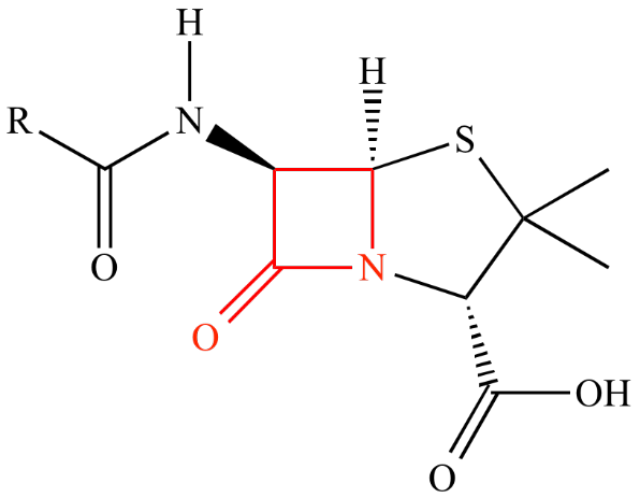

Figure 3. Structure of penicillin with the β-lactam ring highlighted.

The β-lactam ring is a very strained structure, which provides its strength as an antibiotic because the molecule would prefer to break that ring. The ring binds to penicillin-binding proteins (PBPs) anchored in the cell membrane of a bacterium, and this breaks the bond between the nitrogen and the carbon that is double-bonded to the oxygen. This reaction releases the strain of the 4-membered ring. The molecule remains attached to the PBP preventing it from performing its function and disrupting the formation of the bacterial cell wall structure. β-lactam antibiotics are usually prescribed as bactericidal, narrow-spectrum antibiotics that work against gram-positive bacteria, though some exhibit bactericidal activity against gram-negative bacteria. Examples of gram-positive bacteria include *Streptococcus* species, *Staphylococcus* species, and *Clostridium* species.

2b. ii. DNA synthesis inhibition | Fluoroquinolone antibiotics

DNA is necessary for cell growth and cell division, and for a bacterial infection to spread—which can spread fast—a lot of DNA replication needs to occur. There are various steps involved in the replication of DNA, so there are several possible targets for drugs to disrupt this process, but fluoroquinolones and related derivatives are among the most commonly prescribed DNA synthesis-inhibiting antibiotics. To separate the DNA strands of a bacteria for replication, the topoisomerase IV and DNA gyrase enzymes are needed to reduce the strain or tension in the DNA strands. Fluoroquinolones work by entering the bacterial cell and binding directly to topoisomerases which causes a conformational change (change in shape or structure) that prevents the topoisomerase from disconnecting from the DNA. This places such significant strain on the DNA that the strand effectively snaps apart. The DNA cannot reattach, and because the cells need the genetic material for replication and for producing necessary metabolic molecules, the cells die.

As it so happens, DNA does occasionally naturally become damaged and most organisms have evolved mechanisms to repair the damage when needed. If a gap appears in the DNA, signals are sent to genetic mechanisms in the bacteria cell and stimulates the SOS response—a bacterial genome repair mechanism. However, this repair mechanism utilizes what is essentially a lower-quality DNA Polymerase, which creates inaccurate copies of the DNA or may even add extra bases in the DNA, leading to fatal additions or non-functional genes, which can impair the cell's functions and even kill it.

Fluoroquinolones are generally bactericidal, although some are bacteriostatic, and fluoroquinolones can be used against gram-positive and gram-negative bacteria meaning they are broad spectrum antibiotics. Fluoroquinolones should not affect humans because the bacterial enzymes are sufficiently different from human enzymes and human genome mechanisms, but severe side effects of prolonged fluoroquinolone use include tissue damage or toxicities of the central nervous system, cardiovascular system, and the skeletal muscles (Strauchman & Morningstar, 2012). This is hypothesized to be a result of fluoroquinolones acting on mitochondria in human cells. Mitochondria have their own genomes which resemble those of bacterial cells, meaning their DNA can be attacked the same way bacterial DNA is attacked. Extended fluoroquinolone use can be detrimental as a result of inducing mitochondrial dysfunction over time, and suddenly treating something as simple as a urinary tract infection can lead to impaired human function if antibiotics are misused (Lawrence, Claire, Weissig, Rowe, 1996; Hall, Finoff, & Smith. 2011; Kalghatgi et al., 2013; Hany, Jörns, & Rustenbeck, 2014). In fact, this has been demonstrated as true of several bactericidal antibiotics including beta-lactams. Due to this, there have been attempts to address this issue by utilizing antioxidants that specifically protect mitochondria from fluoroquinolones (Lowes, Wallace, Murphy, Webster, & Galley, 2009). Under proper management and adept professional care, however, fluoroquinolones are an effective choice when prescribed for respiratory and urinary tract infections, as well as skin infections, bone and joint infections, and typhoid infections.

2b. iii. Protein synthesis inhibition | Tetracycline antibiotics

Proteins are needed for every metabolic process, responding to environmental chemical gradients, signaling pathways, binding to various surfaces, regulating gene expression, and more. These macromolecules are just as crucial and varied as DNA, and serve many critical roles in the cell. Protein synthesis is the final step in the "central dogma" of biology. First, DNA is transcribed to create RNA, which then is translated into a protein by the ribosomal complex. There are many possible targets in protein synthesis, and as such there are a variety of drugs that target various structures and steps of protein synthesis in bacteria. Tetracycline antibiotics, in particular, bind to the 30s subunit of the bacterial ribosome, which interfere with the RNA binding to the ribosome and can block the translation process altogether. A bacterial ribosome consists of a 50s subunit and a smaller 30s subunit. Human ribosomes are composed of a larger 60s subunit and a 40s subunit, meaning tetracyclines do not interfere with human protein synthesis and are therefore safe for clinical use.

Tetracyclines are generally bacteriostatic, meaning they stop the growth of bacteria, but can also be bactericidal or made more potent with the right serum. Though tetracyclines have been shown to be toxic to mitochondria, tetracyclines are generally considered non-toxic antibiotics. Clinically relevant doses are generally not concentrated enough to cause serious metabolic damage, and because mitochondria 'live' within other cells, they obtain many of their nutrients from their host cell. Mitochondria produce comparatively few proteins and halting protein synthesis within mitochondria might lead to a chemical imbalance, but it can be corrected. Tetracyclines are generally prescribed

for respiratory tract infections, urinary tract infections, gastrointestinal tract infections, skin infections (including acne), and sexually transmitted diseases such as chlamydia, gonorrhea, and syphilis.

2c. Threats of antibiotic resistance.

With the variety of antibiotics available for clinical treatment of various infections, it would seem that bacterial infections are a thing of the past in the modern world. Surely Paul Ehrlich hoped this would be the case and hence dedicated his life to realizing this vision. However, a chemical solution to a problem controlled by genetic factors was not one that could last long. As previously mentioned, antibiotics are naturally produced by soil bacteria that compete with each other by secreting chemicals that will either kill or halt the growth of other bacteria. Those that can produce more potent or more effective antibiotics stand a better chance of surviving and growing, and some even produce multiple antibiotics which gives them a wider range of bactericidal or bacteriostatic activity.

Going on the offensive by producing better or more antibiotics is not the only way bacteria can compete with each other; bacteria can also defend themselves against various antibiotics through several different mechanisms. This is called antibiotic resistance, wherein bacteria develop resistance to certain antibiotics that they were previously susceptible to. Antibiotic resistance is a genetic response to a chemical threat and is not inherently bad because is a natural biological phenomenon. These defenses develop in

two ways: 1) through genetic mutations introduced during replication and growth that alter the structure of various binding sites, or 2) by acquiring whole genes from an external source such as directly from another bacterium, or from the surrounding environment.

In 2014, one study discovered that resistance genes are widespread in non-clinical settings, but also in settings with non-detectable antibiotic molecule concentrations (Nesme et al., 2014). This study examined metagenomic data sets from various biomes such as different types of soil, the ocean, the human gut, Alaskan permafrost, etc. The study found that bacteria in relatively more isolated areas possessed transferrable antibiotic resistance genes despite having no exposure to human-made antibiotics. Though these bacteria were not immediately or constantly exposed to high levels of antibiotics, the genes for resistance had made its way through the environment into remote regions most humans cannot access. That alone should demonstrate concern for the potential impact antibiotic use has and how it may even place humans at greater risk of contracting resistant infections. The study also found that bacteria had more transferrable resistance genes and accumulated them more quickly in stressful environments such as hospitals and the human gut where bacteria may be regularly exposed to antibiotics. These concerns are echoed by preceding papers that confirm simple antibiotic use is the single most important factor driving antibiotic resistance (Aarestrup, Seyfarth, Emborg, Pedersen, Hendriksen, & Bager, 2001; Byarugaba, 2004).

Antibiotic resistance can develop quickly. In 2016, researchers demonstrated the rapid evolution of a wild-type (naturally-occurring) *Escherichia coli* (Baym et al., 2016).

In the span of 11 days, the bacteria evolved to survive concentrations of antibiotic up to 3,000 times greater than what is normally needed to kill the wild-type E. coli. In natural settings where there is an abundance of resistance genes, evolution is more easily facilitated, and even faster in healthcare settings due to the significant selective pressure of antibiotics. This research article provides the first large-scale observation of the adaptive behavior of bacteria as they encounter increasingly higher concentrations of antibiotics and evolve to survive in them. To examine how the bacterium *Escherichia coli* adapts to increasingly higher doses of antibiotics, researchers created a 2'x4' petri dish, divided the dish into 9 sections, and saturated each section with different concentrations of antibiotics. The two outermost sections of the dish had no antibiotic. The next inward section contained only 3 times more than the minimum clinical concentration normally needed to kill the bacteria. Each further inner section represented a 10-fold increase in antibiotic concentration (exponential gradient), with the center of the dish containing 3,000 times more antibiotic needed to kill the bacteria than under normal clinical conditions. In the span of 11 days, the bacteria evolved and grew to fill all sections of the petri dish.

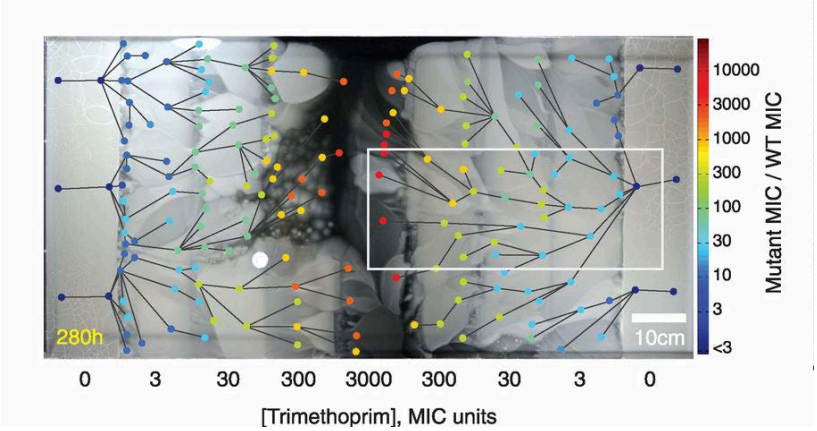

Figure 4. The four-step trimethoprim MEGA-plate after 12 days. *E. coli* appear as white on the black background. The five stages moving inward refer to the 0 MIC units to the 3000 MIC units (3 – $3x10^3$ = 1000x increase), with the lowest concentration on the outer portions of the plate, and the greatest concentration in the middle portion of the plate. (Baym et al., 2016).

It is also important to note that none of the bacteria made the jump from the no-antibiotic portion of the petri dish to the 1,000x antibiotic concentration section in the middle, indicating that this ability to survive greater concentrations of antibiotic is a step-wise progression that can be prevented by using a strong enough antibiotic or strong enough concentration. However, as previously mentioned, using a strong antibiotic for an extended period of time can lead to toxic effects within humans as a result of mitochondrial dysfunction, and there is the risk that simply increasing the dosage could possibly facilitate the step-wise or exponential evolution of resistance in an infectious group of bacteria. Additionally, if the resistance spreads, then even more people will have to use stronger antibiotics, contributing to a positive feedback loop in which the ultimate

conclusion may be a complete inability to treat infections with resistance to antibiotic concentrations too harmful for humans to use clinically. The mere speed at which this can occur, as demonstrated by the MEGA-plate in Figure 4, is what has made many so eager to study and address this issue before it is too late.

2c. i. How antibiotic resistance develops

As previously mentioned, antibiotic resistance develops in two general ways: 1) through genetic mutations that alter the structure of various antibiotic binding sites, or 2) by acquiring whole genes for new structures or metabolic pathways, and these come from an external source such as directly from another bacterium, or from the surrounding environment. There are many published reviews on the complex various mechanisms of resistance, but generally, these are:

1. Bacteria can regulate the permeability of their cell wall which restricts antibiotic access to target sites,
2. Bacteria can actively efflux (pump out) the antibiotic from the cell,
3. Microbes can enzymatically modify the antibiotic into a non-harmful form,
4. Bacteria can acquire or utilize alternative metabolic pathways to those inhibited by the drug,
5. Bacteria can modify antibiotic targets to prevent binding, and/or

6. Bacteria can overproduce the target enzyme to allow normal function which counteracts the inhibitory effects of antibiotic binding.

One of the ways antibiotic resistance develops is through mutation, which is generally a spontaneous, random alteration of an organism's genetic code. The bacteria with beneficial mutations then pass on these mutations to the following generation of bacteria who inherit the new ability or genetic trait. This acquisition of genes from a parental source is referred to as vertical gene transfer. Mutation cannot create whole genes; as such, mutations generally confer antibiotic resistance by altering the binding sites for various antibiotics. For example, bacteria susceptible to β-lactam antibiotics may mutate the gene responsible for the PBP structure. Bacteria that normally succumb to fluoroquinolone antibiotics may alter the structure of a topoisomerase through random mutation. In tetracycline-susceptible bacteria, a mutation could lead to a modified 30s bacterial ribosome subunit that may perform as well or slightly less efficiently, but will in turn survive antibiotic treatment. These modifications could either make the binding sites more likely to bind the respective antibiotic, in which case the mutation is not passed on because the cell will be killed, or the mutation could lead to weaker or no antibiotic binding which is a beneficial trait that will be passed on so long as the modified structure still performs its original function. Alternatively, the structure could be eliminated or replaced with a substitutive structure. Another way mutation can confer resistance is by promoting the production of enzymes (proteins that participate in various metabolic processes and can initiate or catalyze specific reactions) that can inactivate antibiotics or

interfere with the binding site. For example, β-lactamases cleave the bond between the nitrogen and the carbon that is double-bonded to the oxygen in β-lactam rings, which prevents the antibiotic from binding to PBPs and disrupting cell wall synthesis. Yet another way that mutation can increase resistance is by preventing the entry of a drug into a cell altogether. This is particularly beneficial against antibiotics with targets that are within the cell such as fluoroquinolones (DNA) and tetracyclines (protein synthesis). It is important to note that mutation it is somewhat limited in that it does not create entirely new structures or new genes.

If the acquisition of genetic material from parental sources is vertical gene transfer, then the acquisition of genetic material from non-parental sources is referred to as horizontal gene transfer. Horizontal transfer can confer the same types of resistance that vertical gene transfer can, but it is particularly advantageous to bacteria in that whole genes, including genes for new structures or new ways to get rid of drugs, can be acquired this way. There are three general methods of horizontal gene transfer in bacteria: transformation, transduction, and conjugation. Transformation is the uptake of short fragments of naked, 'free' DNA from the surrounding environment. Segments of genomic material can often be found floating freely in the environment, and some bacteria will naturally take up these genes when they encounter a stressor such as low water, high heat, or even chemical stress. For this reason, transformation is a relatively common laboratory research method used to alter or modify bacteria in some way, such as making bacteria glow (as a result of the acquisition of a whole new gene). Transduction refers to the horizontal transfer of genes which are transferred from one

bacterium to another through a bacteriophage—a virus that only infects bacteria. When a bacteriophage infects a bacterium, its genetic information incorporates itself into the bacterial genome, which is then transcribed and translated alongside normal bacterial genes. When assembling new bacteriophages, a portion of the host bacterial cell DNA may be accidentally packaged with the viral genome. When the new bacteriophage infects a different bacterium and injects its genome into that cell with the DNA from another bacterial cell, the new DNA becomes incorporated into the bacterial cell. In this way, whole genes can be shared between different bacteria. The third method of horizontal transfer is called conjugation, and may be the most common method of conferring antibiotic resistance. This method requires direct cell-to-cell contact via a conjugation pilus, or sex pilus, which is a hollow tube formed by the cell donating the genetic material, and this pilus creates a biological tunnel through which genes can move from one cell to another. The bacterial cell donating its genetic material does not lose its resistance; instead, it creates a copy of those genes and transfers the copies to the cell without those genes.

Horizontal gene transfer can create new structures in cells and provide alternative metabolic pathways. One of the most effective antibiotic resistance structures is referred to as a drug pump. These essentially pump out a drug if it enters the cell. While drug pumps do little against antibiotics that target external cellular structures, antibiotics like fluoroquinolones and tetracyclines are more readily pumped out of the cell before they can reach their target sites as a result of these structures. This is particularly dangerous for individuals who contract bacteria that possess drug pumps, as this greatly reduces the

number of possible targets for which the antibiotics can act on. This is spread quickly and easily, posing a serious risk to global health and treatment of disease.

2c. ii. How human use of antibiotics contributes to resistance.

Why does antibiotic resistance matter? Here's a hint: multidrug-resistant bacteria are also commonly referred to as 'superbugs'—a term more often used in news media because it requires significantly less effort to say and is a catchier name. There have already been several waves of extremely resistant pathogens, or superbugs, one of the most recognizable being MRSA, or methicillin-resistant *Staphylococcus aureus*. These superbugs are resistant to the majority of antibiotics and some already have developed resistance to all available antibiotics. For example, early in September of 2016, a Nevada woman in her 70s died after contracting carbapenem-resistant *Enterobacteriaceae* (CRE)—a bacteria that generally causes digestive tract infections. Physicians and researchers tested the bacteria against 26 different antibiotics, and it was resistant to each one, including a drug named Colistin that is used as a potent, last-resort treatment because it causes significant kidney damage (Chen, Todd, Kiehlbauch, Walters, & Kallen, 2017). This particular pathogen is becoming a more pressing issue. Prior to 2012, there were 25 reported cases of this bacteria in the US. In 2012 alone, there were an additional 12 cases of infection by this pathogen.

Recall that penicillin was made available to the general public in mid-1945. However, in the same paper that Alexander Fleming published announcing penicillin, he

also described a group of bacteria that seemed to be unaffected by the antibiotic (Fleming, 1929). In 1940, English biochemist Sir Edward Penley Abraham, CBE FRS (1913 – 1939) and Ernest Chain—the Oxford biochemist who worked with Howard Florey to mass produce penicillin—recorded their discovery of an enzyme isolated from bacteria that was capable of destroying penicillin (Abraham & Chain, 1940). Before penicillin was mass produced for commercial use, a natural defense against it was found, prompting Alexander Fleming to warn of antibiotic resistance in his 1945 Noble Prize acceptance speech (Fleming, 1945). The last thing he relayed in his speech was the following hypothetical scenario as an example of how antibiotic resistance may easily get out of hand:

> Mr. X. has a sore throat. He buys some penicillin and gives himself, not enough to kill the streptococci [a bacterium] but enough to educate them to resist penicillin. He then infects his wife. Mrs. X gets pneumonia and is treated with penicillin. As the streptococci are now resistant to penicillin the treatment fails. Mrs. X dies. Who is primarily responsible for Mrs. X's death? Why Mr. X whose negligent use of penicillin changed the nature of the microbe. *Moral*: If you use penicillin, use enough. (1945)

This is a situation of our own making. Physicians use antibiotics, and sometimes an antibiotic is not strong enough to fight an infection, or not enough is used. Antibiotic

resistance is globally problematic for human health because it means that bacteria become less susceptible to current drug routines. One of the more visible upstream causes is the gross over-prescription of antibiotics; some physicians prescribe up to three times as many antibiotics as their counterparts (Fridkin et al., 2014). Often a physician will prescribe a broad-spectrum antibiotic to cover as many bases as possible, so to speak. This is not uncommon especially because not everyone has the time, or wants, to wait for a lab diagnosis of an infection. Other patients convince themselves antibiotics are the appropriate response to illness, even in cases of a mild, self-limiting infection, and are persistent to the point of harassing physicians for antibiotic prescriptions. This may be due to a lack of effective communication of the risks or a gap in the education. Physicians who overprescribe antibiotics may not be convinced of their contribution to antibiotic resistance, or may care less about educating incessant, ignorant patients and instead prioritize the convenience of prescribing a broad-spectrum antibiotic. Some physicians have pushed back on this reasoning, claiming there is little to no harm in prescribing an antibiotic cocktail to a patient who is visibly infected and for whom a laboratory diagnosis can be obtained the following day (Hoffman, 2012; Ingram, Seet, Budgeon, & Murray, 2012). Recall that bacteria have evolved ways to combat antibiotics, meaning that administering a particular antibiotic while the rest succumb to the same drug makes it easier for the surviving bacteria to grow and multiply (reduced competition), and further spread this resistant character. Suddenly, as Fleming illustrated, human use of antibiotics has “changed the nature of the microbe” and stronger, perhaps more targeted antibiotics are necessary when treating a patient for an infection. Without aggressive action to

compensate for this issue, existing drug routines may become obsolete. In developing countries, a significant number of infections are becoming increasingly multi-drug resistant as a result of similar misuse in field medicine (Byarugaba, 2004). Overuse accelerates resistance.

It is also important to note that the livestock industry uses antibiotics to minimize infections in animals kept in close quarters, and are also often overused due to their secondary benefit of growing animals larger at a faster pace. As a result, resistance can further develop within livestock and downstream of the growth process including livestock consumption. Some bacteria have been isolated from food animals and display resistance to last-resort antibiotics like carbapenems (Fischer et al., 2012; Fischer et al., 2013). However, the scope of this paper is more interested in the healthcare setting, and will thus not delve further into livestock antibiotic use.

Overuse and misprescription, the most significant contributing factors to the spread of antibiotic resistance, are not limited to the doctor's office. Individual consumers need to be aware of how general misuse accelerates this issue. Taking antibiotics for viral infections such as the cold or the flu will not cure the infection; instead, the antibiotics may target bacteria in your body that is beneficial or at least not disease-causing, and promote resistance in otherwise harmless bacteria, which can then be spread through vertical or horizontal gene transfer. Taking another person's antibiotics and taking the wrong antibiotics also contribute in this manner. Another common way that consumers perpetuate antibiotic resistance is by not completing a prescription for antibiotics, instead choosing to stop consuming antibiotics when symptoms disappear. The risk of this line of

thinking is that the antibiotics may have killed most of an infection, at which point most symptoms will disappear, but does not kill all of them. As a result, the consumer falsely believes that all infectious bacteria are killed, and dispose of their antibiotics. Not only do the remaining bacteria likely possess resistance genes which can then be passed on to other bacteria via gene transfer, but the unused antibiotics then end up in the waste stream where more bacteria can be exposed to the antibiotics in concentrations that are too dilute to kill most, instead promoting tolerance to the treatment.

The solution seems clear then: use antibiotics less frequently for less serious infections, take antibiotics only when prescribed, and complete the full prescribed drug routine so as not to introduce antibiotics into the waste stream. While this appears to be a simple fix to a worldwide problem, this is flawed thinking as a result of one major factor: the bacteria generally still retain their resistance genes, so antibiotics will still need to be prescribed for a variety of infections to prevent the further spread of these genes. While antibiotic traits can be lost, the process of losing a gene is much slower than that of gaining a gene. Currently there are not many widespread, inexpensive, and accurate ways to determine the level of resistance a particular bacterium may have or what antibiotics it may be susceptible to. This is not a consumer problem so much as it is a healthcare problem.

2c. iii. How can we address antibiotic resistance?

The next step is to look at the current methods of addressing the problem. Due in part to the groundwork laid by Paul Ehrlich, there is still much funding being poured into traditional drug discovery. The PEW Charitable Trusts is an independent, non-profit, non-governmental entity that creates an annual report based on publicly-available information provided by studies pending approval of drugs. According to the PEW Charitable Trusts, there are 41 drugs in development as of March 2017. Generally speaking, only about 20% of new drugs are approved for use, so only about 5 of these drugs may statistically become commercially available (Hay, Thomas, Craighead, Economides, & Rosenthal, 2014). To compound this somewhat disheartening reality, the antibiotic discovery pipeline is quickly dwindling. As a result, some have focused their efforts on finding new target structures to create novel antibiotics (McDevitt, Payne, Holmes, & Rosenberg, 2002; Rao, De Waelheyns, Economou, & Anné, 2014; Culp & Wright, 2016). However, very few have come to market, including Zyvox™ (linezolid; used to treat skin infections and pneumonia), Cubicin™ (daptomycin; used to treat skin and tissue infections), and Sivextro™ (tedizolid; used to treat MRSA skin infections). For some, this may indicate that the end of the chemical era in the treatment of bacterial infections is coming to an end, unless there is a way to repurpose existing drugs or somehow reverse the acquisition and spread of resistance genes.

It seems there has already been some success in addressing this issue for some infections. One approach is to administer an antibiotic along with an anti-resistance compound to minimize the risk of developing resistance. For example, one commercially

available drug called Augmentin® which is used to treat a variety of infections including sinusitis, pneumonia, ear infections, skin infections, and urinary tract infections, among others. It is a combination of amoxicillin, which is a type of penicillin (β-lactam), and clavulanate potassium. The function of clavulanate potassium should be somewhat obvious from the other drug it is combined with, which is a β-lactam antibiotic; clavulanate potassium deactivates β-lactamases which are enzymes that cleave the β-lactam ring bond between the nitrogen and the carbon with a double-bonded oxygen. This dual action makes amoxicillin-resistant cells susceptible to amoxicillin again which provides the combination drug its wide breadth in treatment. However, if the structure of the PBPs that β-lactam antibiotics would bind to is altered, the effectiveness of Augmentin® is less than if the bacteria simply secreted an enzyme that the clavulanate potassium could deactivate. Additionally, resistance to Augmentin® has already been recorded (Oteo et al., 2008; Rahnama'i, Wagenvoort, & van der Linden, 2009). Regardless, the improved effectiveness of a combination drug provides some hope to the rapidly vanishing antibiotic discovery pipeline.

Despite the emergence of resistance to combination drugs like Augmentin®, there have been attempts to direct their use in a way that minimizes the development and spread of resistance (GlaxoSmithKline, 2006). This has been incorporated into a strategic approach referred to as antimicrobial stewardship programs. These are coordinated programs designed to improve and inform the practice of prescribing antibiotics. The Centers for Disease Control and Prevention (CDC) together with two other national agencies have created a set of guiding principles and general techniques after which other

antimicrobial stewardship programs can be modeled (Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases & Division of Healthcare Quality Promotion, 2017). The CDC recommends that these methods and standards be adopted by all hospitals, long-term care facilities, emergency surgical centers, dialysis centers, and private practices.

2c. iv. Antibiotic resistance diagnosis.

As previously mentioned, a critical factor in the slowing or reversing of antibiotic resistance is the need to use antibiotics more purposefully. This means that the general practice of blindly prescribing broad-spectrum antibiotics or antibiotic cocktails needs to end. To do this, physicians need some way to identify which pathogen is responsible for an infection, and furthermore need to know which antibiotics the pathogen is resistant to. Generally speaking, a physician can interpret symptoms and vital statistics to determine which bacteria or group of bacteria are responsible for a bacterial infection. However, since a variety of bacteria can stimulate similar or identical symptoms, physicians rarely rely solely on medical interpretation and instead opt for antibiotic susceptibility testing (AST).

Laboratory testing is generally a phenotypic AST method that relies on a sample of bacteria collected from an infected area which can then be grown in a medium, typically an agar tube, agar plate, or liquid broth, and then can be physically characterized by experienced microbiologists who then inform the physician. In a

laboratory, a common way to test which antibiotics an infection may be resistant to is to soak small disks in various antibiotics at clinically relevant concentrations and place them onto a freshly spread agar plate. The plate is covered and left undisturbed for a period of time to allow bacterial colonies to grow and cover the plate, and then the plate is checked for the presence of halos. This is called a Kirby-Bauer disk diffusion assay which can be used to test multiple antibiotic compounds at once (Jenkins & Schuetz, 2012). Recall that, as on Alexander Fleming's plate (Figure 2), a clear area around a substance indicates that the substance (antibiotic) is capable of killing the bacterial cells.

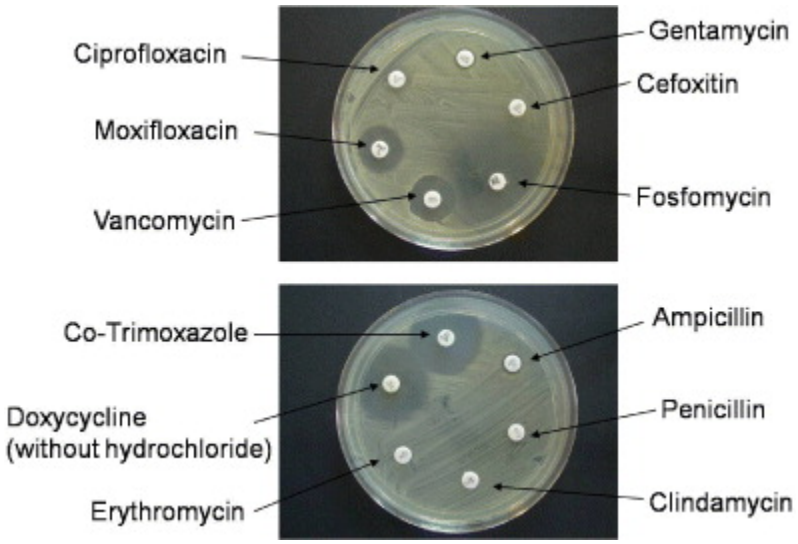

Figure 5. Kirby-Bauer antibiotic resistance assay on a MRSA culture. (Zimmerman et al., 2012).

In Figure 5, the white disks, known as Kirby-Bauer disks, have been soaked in various antibiotics and left on the plate to allow the MRSA culture to grow and respond to the antibiotics (Zimmerman et al., 2012). The presence of the halos, known as zones of inhibition, indicate antibiotic activity against the bacteria, or the bacteria's susceptibility to the antibiotic. The disks with no halo around them indicate that the MRSA culture is unaffected by those antibiotics because it possesses resistance mechanisms against those antibiotics. Again, several days may be needed to confirm which antibiotics are effective against a pathogen. However, as implied in Figure 5, if an antibiotic cocktail does not contain any bactericidal or bacteriostatic antibiotics, the infection will not improve and antibiotic resistance may spread in addition to harming an individual's microbiome and possibly introducing avoidable mitochondrial toxicity. In the case of MRSA which can often be a fatal infection, there may not be enough time to wait for a positive diagnosis or susceptibility treatment.

A modified form of the Kirby-Bauer disk diffusion assay is to use E-tests in place of the antibiotic soaked disks (Jorgensen & Ferguson, 2009). E-tests are strips that are loaded with a gradient of concentrations of individual antibiotics, producing a zone of inhibition on a bacterial plate much like in the disk diffusion assay, allowing physicians to limit the dosage or strength of antibiotic needed for antibiotic treatment of an infection. While this sounds promising, E-test strips cost several USD per individual strip, and each strip only contains one antibiotic, so when testing for susceptibility multiple strips may be needed. This is less than ideal, so current practice is to reserve this diagnostic method for use when a pathogen's identity is certain and minimal tests are needed. This test is also

limited by the amount of time needed for bacteria to grow, but the results are visible at a glance and can quickly inform physicians who wish to limit clinical strength of prescription antibiotics.

Though bacteria can replicate rapidly, a typical phenotypic AST usually requires 24-48 hours of uninterrupted growth to ensure the formation of mature colonies that can be further examined under microscopes or characterized using a number of other tests. This is generally why in modernized healthcare it may take several days to learn the results of a throat or ear swab or blood test, and in this time physicians tend to prescribe broad-spectrum antibiotics or antibiotic cocktails to help alleviate some symptoms with the hope of possibly getting a head start on treatment (Hoffman, 2012). In emergency situations, however, this is not an option to consider, for the sole reason that testing may require more time than is available to the patient. In resource-limited settings, not only may lab access be difficult, but there may be unreliability in the ability to maintain conditions for growth and sterile technique. Additionally, with increasing time between sample collection and reporting results to patients, the likelihood of patients seeking treatment decreases (Pop-Eleches et al., 2011; Taber, Leyva, & Persoskie, 2015). This inefficient step is the first that needs to be addressed in diagnosing an infection. Reducing the time needed between obtaining a sample and tailoring a custom antibiotic treatment plan is important to optimize successful treatment and minimize the risk of antibiotic resistance.

In clinical diagnosis, physicians more greatly value the phenotypic results of ASTs as the phenotypic response is theoretically identical to the response of the pathogen

in question, in which case the results directly translate to prescribed treatment. Following this reasoning, the primary development in ASTs have focused on making phenotypic ASTs more rapid and accurate (van Belkum et al., 2013). The field of microfluidics has risen to meet this challenge, and has produced a number of tests that can work with incredible accuracy and increasingly miniscule amounts of fluid, as the name may suggest (Reece et al., 2016).

Microfluidics has made it possible to obtain phenotypic results in as little as 1 – 3 hours through a category of tests known as flow cytometers or lateral flow assays, in which there is no need for a bacterial culture (Choi et al., 2013; Hou et al., 2014; Kim, Cestellos-Blanco, Inoue, & Zare, 2015). One study published in August of 2017 claims to have created a method that can detect susceptibility in less than 30 minutes (including sample preparation, loading, and processing) using minute concentrations of bacteria (Baltekin, Boucharin, Tano, Andersson, & Elf). Flow cytometers and lateral flow assays generally work by moving cells through a small opening using a small amount of fluid. The small opening allows for the isolation of small amounts of cells or even single cells which can then be analyzed by measuring a change in electrical conductivity or by the detection of light as emitted or scattered by a bacterial cell. A computer analyzes these results and can range in complexity from simple quantification tests to, as mentioned here, susceptibility tests. This sounds as though the problem is solved, but the biggest barrier to their implementation is the cost of individual tests. These tests range in cost depending on the complexity of the analyses, require access to reliable electricity, are not always easily portable, depend on the use of precise mixtures of costly fluids and buffers,

and many have limited shelf life. Evidently, phenotypic ASTs are not yet the optimal choice for rapid, point-of-care diagnosis of antibiotic resistance.

Genotypic ASTs are generally faster than most traditional methods of laboratory detection, providing results in as little as 108 minutes instead of days (Fredborg et al., 2015). Genotypic ASTs utilize common genetic research techniques such as DNA hybridization in which a known genetic sequence can be 'tagged' with a fluorescent probe, or use polymerase chain reaction (PCR) to rapidly amplify small amounts of bacterial genome and detect resistance genes with DNA fingerprinting (gel electrophoresis). There are several issues with relying on genotypic ASTs. In the case of multiple infections in a single patient, there is a risk of false-positive results due to the contamination of specimens. Additionally, clinical diagnosis of genotypic ASTs much be cautiously interpreted because organisms in an individual's normal microbiota may display matching genes. Genotypic ASTs are also of somewhat limited use to physician because possessing resistance genes does not necessarily mean that the bacterium is resistant to an antibiotic; as such, bacteria with resistance genes may not necessarily express any or all resistance genes, and in reality can be susceptible to an antibiotic weaker than what a genotypic AST might indicate (van Belkum et al., 2013). Previously it was discussed that antibiotics can cause mitochondrial toxicity, so wherever a weaker antibiotic can be used, it is advantageous to do so to minimize undesirable side effects. A genotypic test requires knowledge of the genes that need to be identified in a sample, so in the case of an unknown resistance gene or of bacteria with many resistance genes, most genotypic tests are not an optimal choice. This means that genotypic ASTs can only

detect the presence of resistance genes, but cannot inform true susceptibility. Perhaps it is time to focus on improving genetic ASTs. The potential drawbacks of existing genotypic ASTs suggest the field is ripe for disruption and innovation.

3. CRISPR

3a. Introduction to CRISPR

In 1987, a Japanese molecular biologist named Yoshizumi Ishino was researching the *iap* gene which codes for a metabolic protein in *Escherichia coli* bacteria. In an attempt to uncover mechanisms that may affect its expression, Ishino and his fellow researchers decided to sequence genetic regions near wherever the *iap* gene was found. The last paragraph of Ishino's journal publication reads,

> "An unusual structure was found in the 3'-end flanking region of *iap*. Five highly homologous sequences of 29 nucleotides were arranged as direct repeats with 32 nucleotides as spacing. [. . .] Well-conserved nucleotide sequences containing a dyad symmetry, named REP sequences, have been found in *E. coli* and *Salmonella typhimurium* and may act to stabilize mRNA. A dyad symmetry with 14 nucleotide pairs was also found in the middle of these sequences but no homology was found between these sequences and the REP sequence. So far, no sequence homologous to these has been found elsewhere in procaryotes, and the biological significance of these sequences is not known." (Ishino, Shinagawa, Makino, Amemura, & Nakata, 1987).

Repetitive extragenic palindromic (REP) sequences are what Ishino was looking for when his team decided to look at the genetic regions surrounding the *iap* gene, as they regulate gene expression (Stern, Ames, Smith, Robinson, & Higgins, 1984). However, Ishino evidently found something he did not recognize in some of these REP sequences. As Ishino described, these were short, nearly-identical DNA segments that we found together with regular spacing between each of the segments. The dyad symmetry Ishino mentioned means that the genetic material can be read the same back and forth - much like the words 'radar' or 'kayak,' both of which are palindromes. The purpose and function of these never-before-described genetic structures remained a mystery, and were considered largely unremarkable for a short while.

Similar unusual genetic structures were soon described in a wide range of other prokaryotic organisms. In 1992, a Spanish microbiologist named Francisco Mojica (1963 – present) studied these structures in salt-loving archaebacteria and recognized that the structures followed certain patterns and exhibited shared features, indicating that something more intricate was responsible for these seemingly highly-organized, yet randomly-placed sequences being observed in many prokaryotes inhabiting many different environments. By the turn of the 21st century, these structures were so commonly found that there needed to be a term for researchers to more easily communicate findings about them, so in 2002 a team of Dutch researchers headed by Ruud Jansen at Utretch University published a paper labeling these structures as CRISPRs, which stands for clustered, regularly interspaced short palindromic repeats (Jansen, Embden, Gaastra, & Schouls, 2002). Curious as to why so many different

bacteria would exhibit similar patterns of foreign genetic storage, Mojica and his team delved further. Genetic technology had rapidly advanced at this stage, so Mojica's genomic research revealed that the sequences were homologous or analogous to portions of bacteriophage genomes (Mojica, Diez-Villasenor, Garcia-Martinez, & Soria, 2005). Bacteriophage are viruses that specifically attack bacteria, but it wasn't clear why the bacteria would be storing the genetic information of their attackers. Mojica realized that this meant the bacteria were somehow storing portions of viruses they had encountered and were utilizing this to recognize the viruses in future infections – essentially a prokaryotic adaptive immune system. Most of these CRISPR sequences demonstrate an ability to fold into stable DNA or RNA structures and act as regulatory factors for protein synthesis or gene expression. In 2006, another group theorized the hypothetical way in which CRISPR worked, and the following year a separate group experimentally demonstrated that bacteria incorporated the bacteriophage genome into their own genome after successfully fighting off an infection (Makarova, Grishin, Shabalina, Wolf, & Koonin, 2006; Barrangou et al., 2007). In 2008, researchers from the University of Illinois discovered that CRISPR can target DNA and can naturally play a role in limiting horizontal gene transfer (Marraffini & Sontheimer, 2008).

CRISPR-Cas9 is the name of the genetic mechanism that specifically targets DNA. The complex comprises of an RNA-guided endonuclease, Cas9, which creates a break in the double-stranded DNA structure. A single-guide RNA (gRNA) can help the Cas9 target specific loci on the DNA based on the sequence of the gRNA. When a bacteriophage infects a bacterium, the Cas9 catalogs small genomic segments of the

invader's genome into the bacterial cell's own genome. Upon future infection of the same or similar bacteriophage, the CRISPR-Cas9 mechanism can effectively recognize the genetic material of the invader and cleave the genome to counter the infection.

Since the actual mechanism of CRISPR is less important to the scope of this paper, it will not be covered in extensive detail, but several components and steps of the mechanism were progressively uncovered in the following years leading up to the real scientific breakthrough: in 2013, a team comprised mostly of Harvard researchers led by Chinese-American biochemist Feng Zhang (1982-present) demonstrated the use of CRISPR-Cas9, a versatile version of CRISPR, in genome editing (Cong et al., 2013). The potential for using CRISPR was suddenly broadened, and, as a promising new method of genome editing, many were quick to conduct research regarding its use. Only five years have passed since the publication of this technology, and it has already been used to remove malaria from mosquitos, repair and reverse retinal degradation in mice (blindness), treat muscular dystrophy in a mouse with the potential for human application, and reactivate genes in human brain cells to counteract an intellectual disability caused by Fragile-X Syndrome (Gantz et al., 2015; Bassuk, Zheng, Li, Tsang, & Mahajan, 2016; Nelson et al., 2016; Liu et al., 2018). Indeed, the speed at which this technology is developing is threatening to outpace the necessary discussions surrounding its implementation. Many of the ethical discussions around genome editing have already been covered, as genome editing is not a relatively new technology. CRISPR refines and simplifies the previously more precarious option of gene therapy by making genome editing more accessible and more precise, and it is comparatively very cost efficient.

3b. Clinical application of CRISPR

3b. i. CRISPR and detecting antibiotic resistance.

CRISPR-Cas9 is the genetic machinery that can be used for DNA editing, and is the form that will likely dominate media headlines for years to come. While this can be used to individually alter bacterium and perhaps edit out antibiotic resistance genes and confer susceptibility genes in their place, this seems somewhat unrealistic and may place unnecessary strain on an individual patient and the individual's microbiota. Instead, there is an opportunity to use a different form of CRISPR to enhance existing antibiotic drug therapy.

CRISPR-Cas13a is comparable to CRISPR-Cas9 in its use for genome editing; however, while CRISPR-Cas9 alters DNA, CRISPR-Cas13a operates on RNA. This is advantageous because changes to RNA are less permanent and therefore potentially less dangerous than editing DNA. One paper published in April of 2017 has recently gained significant interest for their development of a CRISPR-Cas13a mechanism that has proven to be the most sensitive AST yet with attomolar sensitivity—that is $1x10^{-18}$ moles of RNA per liter of liquid—and it is classified as a nucleic acid diagnostic (genotypic) AST (Gootenberg et al.). The CRISPR-Cas13a has two RNA cleaving capacities. First, it is able to cleave RNA that matches the sequence of the customized tracrRNA and gRNA. Once the Cas13a complex finds and cleaves its target RNA, its second cleaving activity is activated. This cleaving is non-specific and far more generic, cleaving any nearby RNA in the cell or in a given reaction mixture. In 2016, the Cas13a complex by itself was

found to be capable of cleaving at least $1x10^4$ times as many general RNA for just 1 target RNA cleaved (East-Seletsky et al.). This cleaving activity produces a strong signal that makes it a powerful diagnostic tool with a potential to detect even trace presence of antibiotic resistance, with picomolar sensitivity—that is $1x10^{-12}$ moles per liter of liquid.

Gootenberg et al. took this naturally existing sensitivity and sought to enhance it for diagnostic use, and upon success dubbed the system SHERLOCK (Specific High-Sensitivity Enzymatic Reporter UnLOCKing) (2017). To obtain the attomolar sensitivity, Gootenberg et al. utilized a genetic technique known as recombinase polymerase amplification (RPA) which is comparable to PCR. Traditional PCR requires cycling between high and low temperatures to produce many copies of genome segments, whereas RPA uses enzyme mixes that can perform a similar genomic amplification within a temperature range of 37°C – 42°C (normal human body temperature is approximately 37°C). This means that no expensive machinery is required to precisely cycle an enzyme mixture between various temperatures, reducing both the cost and the reaction time, and this lends the ability to run many simultaneous diagnostic tests. The RPA increased the concentration of the target RNA in solution, thereby increasing the level of CRIPSR-Cas13a target RNA-specific cleaving activity. This cleaving activity also enhanced the level of non-specific RNA cleaving activity. To utilize this as a diagnostic tool, Gootenberg et al. attached a fluorescent probe to the tracRNA that, when cleaved, activates the fluorophore and produces a visible fluorescent signal, and this activation occurs when the Cas13a cleaves it during non-specific cleavage (2017). The

researchers were able to successfully use SHERLOCK to detect minute concentrations of Zika virus and Dengue virus.

Does this mean that CRISPR-Cas13a can be used as a diagnostic tool to combat antibiotic resistance? Yes—and no. It is able to detect the presence of antibiotic resistance genes, and at incredibly small concentrations which is a critical improvement in early diagnosis. CRISPR-Cas13a solves the issue of signaling the possession of genes by instead indicating active transcription, and therefore existence, of resistance mechanisms within a cell. However, at the time of writing, SHERLOCK suffers from some of the same issues as existing genotypic ASTs: it does not inform physicians of phenotypic action, cannot provide accurate quantification of resistant bacteria, and cannot indicate antibiotic susceptibility thresholds on its own. Additionally, the increased sensitivity of the test places increased pressure towards practicing sterile technique, because even the slightest contamination may indicate a false positive. This whole paper has led up until this point, and suddenly it seems as though it was all for naught. But not so: the critical factor identified early on in this paper was the inability or inefficiency of testing for antibiotic resistance. This technology indicates what is now achievable, and the increase in detection sensitivity with shortened detection time marks the beginning of combating antibiotic resistance. Specifically, it shortens the time between sample collection, diagnosis, and treatment prescription, with incredible accuracy so as to allow patients to obtain a tailored plan within a short period of time at extremely low cost. This alone may significantly reduce the need for broad-spectrum antibiotics and antibiotic

cocktails, and may potentially replace existing phenotypic tests for pathogen characterization.

The authors of the SHERLOCK paper also claim they were able to lyophilize (freeze-dry) both the CRISPR-Cas13a and the RPA enzyme mixture for reactivation at a future time. This is appealing not only for reducing the need to synthesize new SHERLOCK components, but also indicates that it can easily be stored for an extended amount of time with minimal degradation which demonstrates improved shelf life. These confer to it a potential advantage in healthcare settings, and a clear advantage in resource-limited settings where proper storage and long shelf life can be challenging. Phenotypic ASTs remain the best chance at identifying antibiotic resistance, but clinical application of CRISPR is still in its infancy. Because it is a CRISPR-based diagnostic system, part of its value lies in the fact that it can be quickly customized with ease for use in various geographical regions or new pandemic diseases, or can be used to determine the presence of superbugs before they arise in healthcare settings. Such a technology may prove invaluable in the fight to curtail the progress of antibiotic resistance. This alone may not be enough, however, and it is worth discussing where CRISPR technology may lead.

3b. ii. CRISPR and potential antibiotic therapy.

The appeal of a CRISPR-based treatment has driven recent research and discussion surrounding its implementation in a familiar form. Specifically, one of the more hyped concepts in scientific and news media is the idea of the CRISPR Pill. With

knowledge of CRISPR's customizability, there is hope in the scientific community that a CRISPR-based antibiotic may one day replace the reliance on biochemical antibiotics.

A 2014 study reported the manufacture of bacteriophages that carried DNA similar to the antibiotic resistance genes (Citorik, Mimee, & Lu). When these phages infected bacteria, cells registered the DNA as foreign invader genes and the inherent CRISPR-Cas system in the bacteria attacked its own genome. This resulted in the killing of bacteria with only the bacterial resistance genes, and left all other cells lacking the genes untouched. If there were unharmed bacteria following this initial treatment, this was due to the lack of resistance genes and could be effectively treated with existing antibiotics. This alone demonstrates a far greater effect than detection alone, as this CRISPR treatment enhances existing antibiotics or circumvents them altogether.

However, phage therapy still carries its risks, including the potential to cause cancer if an oncogenic bacteriophage is used, or if the immune system recognizes the foreign invader and mitigates the potential desired impact of the treatment (Loc-Carrillo & Abedon, 2011). Additionally, bacteriophages are specific with regards to which bacteria they infect, and searching for an optimal vehicle for CRISPR-based treatment for specific bacteria may prove a costly and time-consuming task that hinders the efficiency of the treatment. However, this may aid in minimizing mitochondrial toxicity.

Another challenge to CRISPR-based phage therapy is that bacteriophages typically degrade in the stomach. To ensure optimal distribution transport of the bacteriophages, if taken orally instead of injected, the bacteriophage would need to be

coated in a lipid layer which can then attach to specific cells where the bacteriophage could then infect those cells. This seems too unwieldy, so MIT scientists proposed and published a proof-of-concept in which they demonstrated the creation of lipid nanoparticles that could cloak CRISPR-Cas mechanisms without the need for viruses (Yin et al., 2017). These lipid nanoparticles are able to fuse with or pass through cell membranes and deliver the CRISPR-Cas machinery directly into the cell. The researchers dubbed this RNA medicine. There is much to be seen in the way of development with this technology, but bypassing the need for use of a bacteriophage possibly inches progress closer to the formulation of an actual magic bullet.

These are baby steps, but they are giant baby steps towards a future that will see a radical shift in the approach to medicine. There is yet a long way to go, and the young age of this powerful technology should warrant some caution surrounding its use, but so far it appears that results are hopeful. Perhaps one day, patients will receive instantaneous results diagnosing infections and several minutes later receive custom medication that specifically targets pathogens leaving the existing microbiota unharmed.

3c. A CRISPR cautionary case.

Despite the optimism surrounding the use of CRISPR-Cas9 as a potential basis for medical treatment and genetic remedy, there ought to be caution regarding its healthcare implementation. Its primary hurdle is that the technology's young age means long-term effects must still be carefully monitored. It was previously mentioned that a team of

scientists reversed blindness in mice using CRISPR-Cas9, the DNA editing CRISPR mechanism (Bassuk et al., 2016). At the time of writing, this study has been cited nearly 70 times. In August of 2016, another team, including the leading author of the previous study, used a similar method to successfully reverse the effects of retinitis pigmentosa: a related genetic disease that causes a loss of vision (Wu et al., 2016). This was reported as a success and additional hope was lent to the potential use of CRISPR as a treatment for eye disorders. Approximately 1 year later, however, the authors of this second study submitted a letter to the editors of the *Nature Methods* journal (Schaefer et al., 2017). In this letter they revealed that they observed more than 2,000 undesired mutations, some cancerous, in each of the mice they had treated, which is certainly not an insignificant number.

The immediate reaction of some media was to suddenly claim that it was far too risky a technology to use, and that CRISPR was dead. Much of the scientific community was appalled and confused with the team's reporting which seemed to stand at odds against the history of CRISPR technology. In response to this potentially devastating letter, many rushed to discredit it, focusing on the team's small sample size, their method of choice for delivering CRISPR to target cells, and even doubted whether it was a peer-reviewed study (Lareau et al., 2017). This illustrates 2 things: 1) the hostility of some in the scientific community towards contradictory findings in CRISPR research, and 2) that more research needs to be conducted to explore the technology before extensive use in humans. As previously mentioned, the study in which scientists reversed blindness in mice has been cited nearly 70 times at the time of writing. If this treatment has similar

downstream effects as the retinitis pigmentosa treatment, a large number of studies have cited a dangerous experiment as support for other potential treatments that may similarly cause cancer. Of course, there is the possibility that the authors of the letter to the editor of *Nature Methods* are incorrect and had practiced flawed procedure, in which case CRISPR-Cas9 as a genetic technique is still considered relatively safe, but even this scenario highlights the importance of exercising the utmost caution in utilizing the technology and paying it the respect it demands.

Treating a non-lethal vision impairment should not lead to the acquisition of a cancer diagnosis, and pretending the findings may not be muddy is a sure way to inhibit or prevent its implementation in the healthcare setting. Additionally, the opinion and perception of the public is a powerful force that can easily result in the rejection of such a technology if ignorance regarding its safety and efficacy spreads quicker than intended. Hence the need to focus on an *ex vivo* application of CRISPR-Cas13a instead of hurriedly rushing into an exciting frontier with CRISPR-Cas9 at the needless cost of even one human life. This preserves the possibility that CRISPR may one day produce Ehrlich's much sought-after magic bullet that in the years and decades to come.

4. How the global community ought to proceed in using CRISPR to combat antibiotic resistance.

Having examined several of the possible uses of CRISPR and briefly touched on challenges to its implementation, it is important to establish how we ought to approach the problem of antibiotic resistance in the genetic era. Although CRISPR can be used by private individuals, it is not recommended, and will likely not place much of a dent in the issue of antibiotic resistance. Remember, it is easier to promote antibiotic resistance than to reverse it, so while an individual can contribute significantly to its spread, an individual will likely not resolve the issue very quickly. As such, this section will focus on larger-scale application of the technology and preach caution, both in healthcare settings and in global field settings.

Recall that diagnostic testing is a key factor in limiting the accuracy and pointedness needed in treating bacterial infections and reducing antibiotic resistance. Most individuals who become sick do not have time to wait for lab results from the doctor's office and as such doctors will prescribe antibiotic drug cocktails which are a mix of various broad-spectrum and narrow-spectrum antibiotics that can tackle a variety of the most common infections. However, in cases of antibiotic resistance this may not always work and may weaken the individual immune system by reducing bacterial competition from beneficial or harmless bacteria, placing the individual at greater risk. The CRISPR-Cas13a/C2c2 (SHERLOCK) mechanism previously described may be the

answer to this issue. Optimistically, this is the early integration of CRISPR in the healthcare setting as a diagnostic technique. This application is considered relatively safe, as it is an *ex vivo* RNA identification technique, requires only a small sample of blood from the infected individual, and significantly reduces both the time and cost of diagnostic testing such that it promotes more individualized prescription and treatment in the same medical appointment. The cost savings largely come from its ability to be mass produced rather quickly and in large quantities, as well as the benefit of isothermal amplification. Part of its appeal lies in the fact that it can be easily customized for use in various geographical regions or pandemic diseases, can be lyophilized, and can easily be stored for an extended amount of time which demonstrates improved shelf life; all of these factors of a CRISPR-based diagnostic test provide it clear advantages over current diagnostic methods used both in the field and in modern healthcare settings. There is little opposition or discussion surrounding this particular application, but that is in part because it is still being studied. However, I still contend that it is not yet ready for global application as it still suffers from issues that plague existing genotypic ASTs, and as such further development is needed. Despite this, its application, distribution, and remarkable specificity paint a promising future for a new class of genotypic ASTs and for combating antibiotic resistance worldwide.

Though there is a temptation to view the CRISPR Pill as another magic bullet, it is a much riskier treatment, and is something that is not yet implementable. This can be discussed in two parts: 1) a pill that compliments or supplements existing antibiotics, or 2) an ingestible or injectable CRISPR-based treatment that replaces an antibiotic

treatment. If the CRISPR pill shows real promise, the first step will likely be to use it in a fashion similar to that of combination drugs like Augmentin®. This may not be made available on the market very soon in part because it does carry the risk of mitochondrial dysfunction (toxicity). However, should future studies uncover clinically relevant doses administered with minimal negative effects in combination with existing antibiotics, this form of CRISPR pill will be the first commercially available option because this does not interfere with the human genome. The other option with an ingestible CRISPR-based treatment is to replace antibiotic treatment, but exactly what that might look like remains to be seen. Targets have yet to be identified, and the method of bacterial cell killing has not yet been detailed. Perhaps one option would be to incorporate synthetic genes into the human genome to combat certain bacterial infections, although the structure of such a gene is yet to be hypothesized and ethical discussions may prevent this from becoming an option. Additionally, phage therapy can be costly, although this may be relatively less so when compared to current phage therapies. Recall Fleming's warnings surrounding the use of penicillin, and the discovery of anti-CRISPR genes, and suddenly it is obvious that resistance to a CRISPR-based treatment can be much deadlier and possibly be the end of our ability to address antibiotic resistance. The CRISPR pill, if not extensively researched for downstream effects, may end up hastening the pressing global issue at hand. For this reason, I suggest that the path forward avoids a CRISPR-based treatment until several years of research can be conducted surrounding its safety, disposal, and downstream effects.

As CRISPR-based technologies advance and phase into medical care, healthcare facilities must adapt their antimicrobial stewardship programs. Some may be resistant at first, instead opting not to use CRISPR-based treatment in favor of existing ones, but eventually the state of healthcare will have to evolve, because without large-scale effort to address the largest contributing source towards antibiotic resistance, bacteria will prevail. The state of research on the CRISPR-Cas system is not yet where it needs to be to create Paul Ehrlich's ultimate magic bullet; however, with the rapid pace of development and the incredible efficiency of the CRISPR-Cas mechanism, a magic bullet may not be too far out of reach. Bacteria are not waiting, and neither should we, but we ought to exercise extreme prejudice and err on the side of caution to avoid a potentially more terrible fate than what we now face with antibiotic resistance.

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