THE EMERGENCE OF ANTIMICROBIAL RESISTANCE GENES OVER THE YEARS AND THEIR GEOGRAPHIC BIAS

By

Deepanwita Chakraborty
21176002

A thesis submitted to the Department of Mathematics and Natural Sciences
in partial fulfillment of the requirements for the degree of
Masters of Science in Biotechnology

Department of Mathematics and Natural Sciences
Brac University
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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing a degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.
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Deepanwita Chakraborty
21176002

Approval

The thesis titled the emergence of antimicrobial resistance genes over the years and their geographic bias submitted by

1. Deepanwita Chakraborty (21176002)

Summer 2022 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Masters of Science in Biotechnology in September 2022.

Examining Committee:

Supervisor:
(Member)

_____

Iftekhar Bin Naser, PhD
Associate Professor, Department of Mathematics and Natural Sciences
BRAC University

Program Coordinator:
(Member)

_____

Iftekhar Bin Naser, PhD
Associate Professor, Department of Mathematics and Natural Sciences
BRAC University

Departmental Head:
(Chair)

_____

Prof. Dr. A F M Yusuf Haider
Professor and Chairperson, Department of Mathematics
and Natural Sciences
BRAC University

Ethics Statement

No animals or living things were used in this study.

Abstract

Antimicrobial agents have played a very intrinsic role in human and animal health since the beginning of the world. However, our irresponsibility has made this life saver, equally deadly. This study shows how much AMR has increased temporally and spatially in only certain bacteria. This might be a way to understand which bacteria acquire more resistance and seek a plausible cause and solution. After using an online database to extract the required sequences and annotate them via various bioinformatics tools, the results were tabulated and presented in graphs. According to timed data, the number of AMR genes increased by more than 2-3 folds in bacteria found in open environments, and with the bacteria encased in the host body, there were no noticeable changes. According to global statistics, AMR has increased higher in underdeveloped nations than in developed ones. For all periods, the average AMR gene count in developing nations was over twice that of developed nations for *E. coli* others exhibited data that was marginally higher for developing nations. However, the variation in the AMR for developing and developed count was not significant for bacteria rarely found in the environment. Thus, we can make a point that bacteria mostly found in open environments and poor countries acquired the most resistance towards antimicrobial drugs than found inside living bodies and richer ones.

Keywords: Antimicrobial resistance (AMR); bacteria; environment; host body; antibiotics; temporal; spatial.

Dedication

Dedicated to boosting awareness about the perturbing case, escalated antimicrobial resistance can be easily subdued by our accountable endeavor.

Acknowledgement

Predominantly, I would like to show my gratitude to Lord Brahma for making me capable enough to complete this project. I would also like to shower my overwhelming thanks to my parents for leading my path and constantly supporting me in my decisions.

I am highly indebted to my supervisor Iftekhar Bin Naser, Ph.D., Associate Professor, Department of Mathematics and Natural Sciences, School of Data Science, BRAC University, for his immense cooperation and dedication and for providing valuable suggestions and comments whenever it was required. I am highly indebted towards Professor Aparna Islam, Department of Mathematics and Natural Sciences, School of Data Science, BRAC University for her unconditional support at all times. My gratitude goes to my mentor Tushar Ahmed Shishir for his complete guidance throughout the study. My special appreciation is to Faria Akhter, Nabonita Chakraborty, Ebtesam, Eera Ashrafy, and Tahsin Shahrin Khan who helped me with full dedication throughout my work.

My honor and respect belong to Professor A F M Yusuf Haider, chairperson, Department of Mathematics and Natural Sciences, School of Data Science, BRAC University, and late Professor A. A. Ziauddin Ahmed, former chairperson, Department of Mathematics and Natural Sciences, School of Data Science, BRAC University for their invaluable support that has influenced me to work in my desired field efficiently.

I would also like to acknowledge our lab officers for their teachings and all lab staff for without their help and support my laboratory work would not have been possible.

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List of Acronyms

AMR	Antimicrobial resistance
E. coli	*Escherichia coli*
P. aeruginosa	*Pseudomonas aeruginosa*
M. tuberculosis	*Mycobacterium tuberculosis*
S. enterica	*Salmonella enterica*
S. pneumoniae	*Streptococcus pneumoniae*
S. aureus	*Staphylococcus aureus*
P. notatum	*Penicillium notatum*
HGT	Horizontal gene transfer

Chapter 1

Introduction

1.1 Background

Providently, one of the most effective chemotherapies in medical history is antimicrobials, said Aminov (2010).[1] It is unnecessary to question how many lives they have saved and how much they have helped in controlling infectious diseases, which for most of human history were the main causes of morbidity and mortality in humans. The antibiotic discovery was bliss for the living world and due to World War II, the United States played a significant part in the development of the drug's large-scale production, turning a life-saving substance with a limited supply into an extensively prescribed medication. After first putting antibiotics into clinical use in the 1940s, they were quite effective in getting rid of deadly bacteria, which led many people to think that infectious diseases would soon become bygones and disappear from all human populations, Aminov (2009)[2] described. Unfortunately, due to the evolutionary and ecological processes in microbial ecosystems, antimicrobial overuse, and abuse, these bacteria have grown resistant to most antibiotics. Currently, finding an antibiotic for one particular bacterial stain that is completely susceptible has become a burning issue. Consequently, it has become a very intrinsic job to find out where and which strains are acquiring more resistance. This will prove useful in seeking the possible reasons and ways to stop them from achieving resistance. Presenting the trend of some selected bacteria acquiring antimicrobial resistant (AMR) genes over time and seeking how the resistance pattern differs from richer to poorer countries, will give an idea of the possible causes and extent of the emergence of AMR genes in bacteria.

1.2 Research aim and objective

The prime purpose of this study is to find out the quantity of AMR genes present in 6 specific bacteria and how they emerged in the past 20 years. Furthermore, finding out how the amounts differ according to their geographical position.

Chapter 2

Literature review

Although people have used antibiotics to cure ailments for millennia, they were unaware that bacteria were to blame until around a century ago. Some of the earliest civilizations used various molds and plant extracts to cure diseases; the ancient Egyptians, for instance, applied moldy bread to infected wounds. But up until the 20th century, bacterial diseases that we now take for granted, including pneumonia and diarrhea, were the leading cause of mortality for people in the industrialized world.

2.1 Antibiotics discovery

Scientists didn't start to see antibacterial compounds in action until the late 19th century. German doctor Paul Ehrlich discovered that some bacterial cells were colored by specific chemical dyes but not others. He concluded that it must be able to develop compounds that may kill specific germs selectively without hurting other cells following this idea. He discovered in 1909 that a substance known as arsphenamine might effectively treat syphilis. Although Ehrlich himself referred to his discovery as "chemotherapy"—the employment of a chemical to cure a disease—it was the first modern antibiotic. Over 30 years later, the Ukrainian-American scientist and microbiologist Selman Waksman, who discovered over 20 antibiotics throughout his lifetime, used the term "antibiotics" for the first time.

Penicillin was accidentally discovered by Alexander Fleming, who appears to have been a little disorganized in his work, said *Microbiology Society* (n.d.)[3]. In 1928, after returning from a vacation in Suffolk, he discovered that a culture plate of Staphylococcus bacteria had become contaminated by the fungus Penicillium notatum. Everywhere the fungus developed on the plate, there were areas free of germs. Fleming separated the mold and raised it in sterile culture.

He discovered that P. notatum was less toxic than the disinfectants in use at the time and proved to be exceedingly effective even at very low concentrations, inhibiting Staphylococcus growth even when diluted 800 times.

Collaborations with British pharmaceutical companies enabled the mass manufacture of penicillin (the antibiotic compound produced by P. notatum), following early studies in the treatment of human wounds. Many survivors of a fire in Boston, Massachusetts, where almost 500 people perished, got skin grafts that are susceptible to Staphylococcus infection. Because penicillin was such an effective treatment, the US government started funding its widespread manufacture. Penicillin was widely utilized to treat infections in soldiers by the time of D-Day in 1944, both in the field and in hospitals across Europe. Penicillin was known as "the wonder medication" and had saved many lives by the conclusion of World War II.

2.2 Antimicrobials and antibiotics

Britannica (2021) [4] wrote that antibiotics are a chemical compound that is toxic to other bacteria and produced by a living thing, usually a bacterium. In a complex environment like soil, organisms undoubtedly use antibiotics to regulate the growth of rival microbes. Bacteria and fungi are microorganisms that produce antibiotics that help prevent or treat disease. The organisms that produce antibiotics have antibiotic/ antimicrobial genes in their DNA. Some organisms uniquely can acquire resistance by taking in the plasmid (extrachromosomal DNA) containing antimicrobial genes. This is one of the reasons they become resistant to a certain antimicrobial.

2.3. Gram-positive and negative bacteria

Gram staining is an empirical technique used to categorize bacterial species into two major groups (Gram-positive and Gram-negative) based on the chemical and physical characteristics

of their cell walls. The technique bears the name of its creator, Danish scientist Hans Christian Gram (1853–1938), who devised it in 1884. It is inconceivable to stress how crucial this finding is to correctly identifying bacteria because all phenotypic approaches start with this assay.

In light of this fact, as explained by Sandal (2004)[5] Gram dried a lung sample smear and covered it with "anilinegentain violet solution of Ehrlich" (also known as Gentian (crystal) violet). Following rinsing with water, Gram added Lugol's solution, a mordant made of potassium triiodide in water. After this, Gram washed the stain away with ethanol. Gram made numerous observations that led him to the conclusion that some bacteria, like *Streptococcus pneumoniae*, maintained their purple color while other species did not (that he termed a negative reaction).

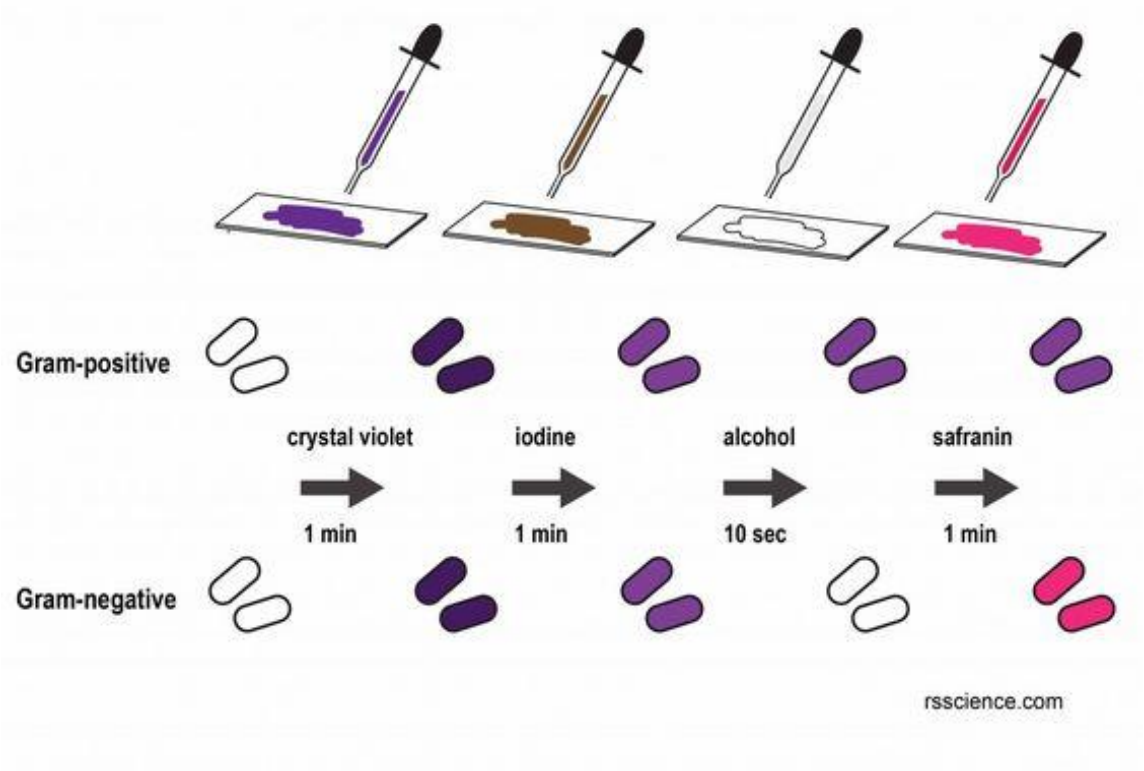

Figure 1: Gram staining method displayed for both Gram-positive and Gram-negative bacteria

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According to subsequent theories, the variation in cell wall composition between the two "groups" was what caused the response. In comparison to Gram-negative bacteria, the bacteria

that maintained the stain (referred to as Gram-positive bacteria) contained more peptidoglycan and fewer lipids. While the solvent dehydrates the thicker cell walls of Gram-positive bacteria, preventing any diffusion of the violet-iodine complex, which seals the pores of the cell and preserves the stain, the solvent had the opposite effect on Gram-negative bacteria, dissolving the lipid layer in the cell wall and causing the crystal violet to seep out.

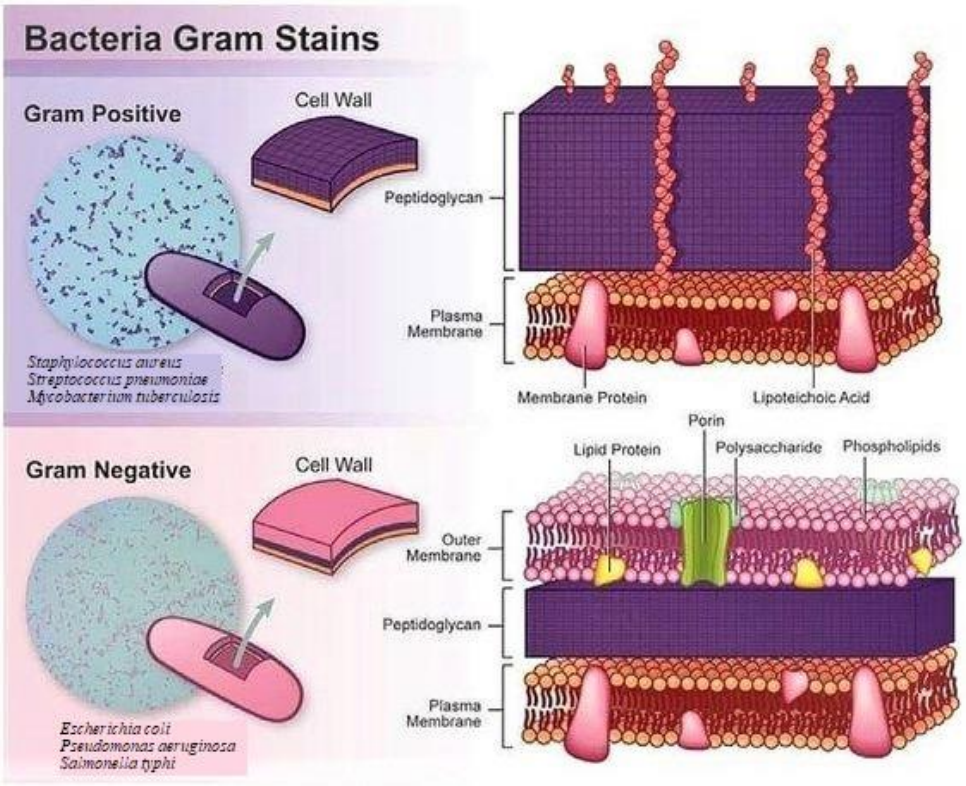

Figure 2: Gram-positive bacteria and Gram-negative bacterial cell wall

2.4 Bacteria included in the study

Escherichia coli

Escherichia coli (*E. coli*) is a facultative anaerobic, gram-negative rod-shaped bacterium. Theodor Escherich published the first description of this bacterium in 1885. The majority of *E. coli* strains are a typical part of the flora in the gastrointestinal tracts of both humans and animals. But some *E. coli* strains have developed into harmful varieties by acquiring virulence

components via plasmids, transposons, and bacteriophages written by Lim, Yoon and Hovde (2010)[6]. Anastasi (2012)[7] stated that presence of *E.coli* in waste water and reservoirs human/animal-impacted environment.

Figure 3: Escherichia coli under electron microscope

Pseudomonas aeruginosa

According to Diggle, Whiteley, (2020)[8], Gram-negative opportunistic pathogen Pseudomonas aeruginosa serves as a model bacterium for research on virulence and bacterial social characteristics. It can be easily discovered in practically every area that has been influenced by people or animals, even though it may be isolated in small amounts from a wide range of settings, including soil and water. Potential reservoirs of *P. aeruginosa* that are resistant to antibiotics can be found in hospital restroom water that eventually drains into bodies of water. An infection with *P. aeruginosa* can harm many different tissues, including the heart (endocarditis), respiratory tract, central nervous system, ear (including external otitis), eyes, bones, urinary tract, gastrointestinal tract, and leather. According to reports, *P. aeruginosa* septicemia had a death rate of 80%.

Figure 4: Pseudomonas aeruginosa under electron microscope

Staphylococcus aureus

A gram-positive bacteria called Staphylococcus aureus is responsible for a wide range of clinical illnesses. According to Tailor and Unakal, infections brought on by this virus are frequent in both community- and hospital-acquired settings (2022)[9]. A kind of gastroenteritis with a quick onset of symptoms, staphylococcal food poisoning is brought on by the bacterium Staphylococcus aureus said Aydin, Sudagidan, Muratoglu (2011)[10]. *S. aureus* is frequently found in the environment (soil, water, and air), as well as in human skin and noses. According to Tong et al. (2015)[11], staphylococcus aureus is a significant human pathogen that is responsible for a variety of clinical illnesses. Along with osteoarticular, skin and soft tissue, pleuropulmonary, and device-related infections, it is a major contributor to bacteremia, infective endocarditis, and these other conditions.

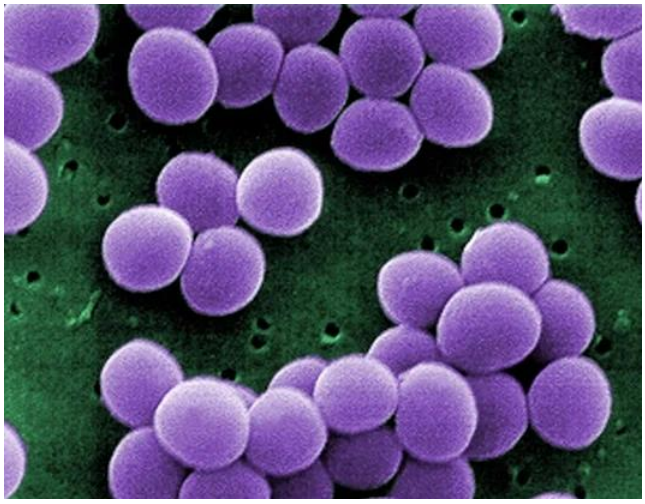

Figure 5: Staphylococcus aureus under an electron microscope

Salmonella enterica

Salmonella enterica is a rod-shaped, gram-negative enterobacterium that causes human diseases ranging from mild gastroenteritis to serious systemic infections, according to Wendy et al. (2015) [12] in their book. Salmonella must grow intracellularly in macrophages for an infection to take place. Fowl typhoid (FT), caused by Salmonella enterica serovar Gallinarum (SG), is an acute septicemic disease of chickens and other galliforme birds[13].

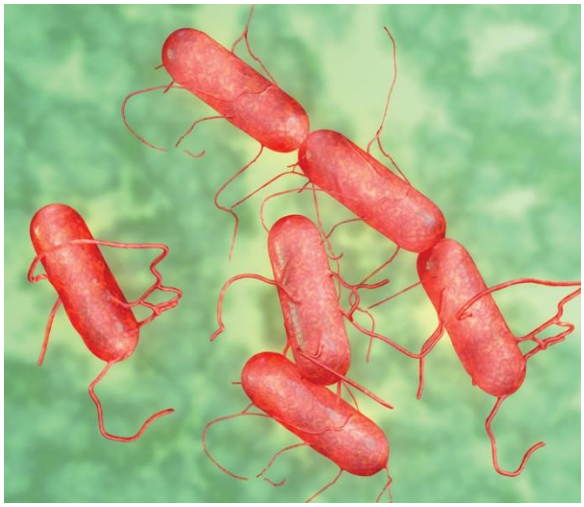

Figure 6:Salmonella enterica under electron microscope

Mycobacterium tuberculosis

A rod-shaped, non-motile, weakly gram-positive bacterium called Mycobacterium tuberculosis. It is an obligate aerobic as well as a facultative intracellular parasite. This clarifies why tuberculosis often affects the lungs as a disease. A person contracts tuberculosis (TB) by breathing in microscopic droplets from an infected person's cough or sneeze. Although it mostly affects the lungs, it can also harm the stomach (abdomen), glands, bones, and neurological system. [14]

Figure 7: Mycobacterium tuberculosis under an electron microscope

Streptococcus pneumoniae

Pneumococcus, also known as Streptococcus pneumoniae, is an aerotolerant anaerobic species of the genus Streptococcus that is spherical, gram-positive, and capable of alpha- or beta-hemolysis. They do not produce spores, do not move, and are typically seen in pairs. In healthy carriers, Streptococcus pneumoniae normally colonizes the nasal cavity, sinuses, and respiratory system without causing any symptoms.

Figure 8: Streptococcus pneumoniae under an electron microscope

2.5 AMR acquirement

When antibiotics are used excessively or when they are not necessary, multidrug-resistant organisms grow. Only a few germs may initially resist antibiotic therapy. Antibiotic resistance is more likely to develop the more frequently antibiotics are used. These MDROs have the potential to infect humans. Large doses of antibiotics administered to humans, farm animals, and even fish in aquaculture led to the selection of pathogenic bacteria that were resistant to a number of different medications. Bacteria may develop multidrug resistance by one of two ways. Firstly, each gene those codes for drug resistance can accumulate many times within a single cell of these bacteria.[15] This buildup often takes place on resistance (R) plasmids. Secondly, increased gene expression for multidrug efflux pumps, which extrude a variety of medicines, may result in multidrug resistance.

Microorganisms have developed additional types of resistance mechanisms due to the prolonged use of many medications, which has resulted in multidrug resistance. There are many persuasive evaluations on this subject Alekshun & Levy (2007) [16]; Ayukekbong et al. (2017) [17]; Choudhury et al. (2012) [18]; Colodner et al. (2004) [19]; Gashaw et al. (2018) [20]; Nikaido

(2009) [21]; Tanwar et al., (2014) [22]. These resistance mechanisms, according to Alekshun and Levy (2007) [23], include novel penicillin-binding proteins, enzymatic drug modification mechanisms, mutant drug targets, increased efflux pump expression, and altered membrane permeability (Alekshun & Levy, 2007) [23]. Bacterial antibiotic resistance genes can spread by a variety of horizontal gene transfer pathways.

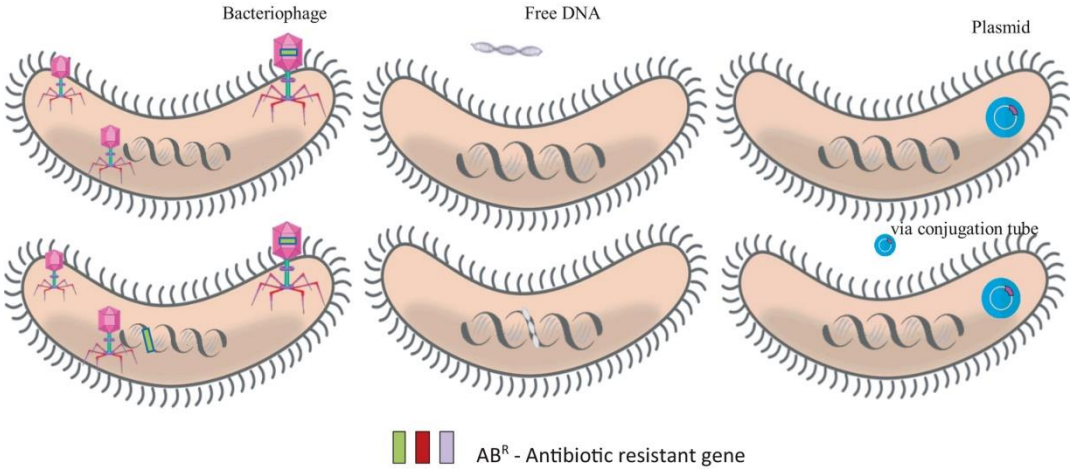

Figure 9: : Acquisition of antibiotic resistance—the mechanism of horizontal gene transfer between different bacterial populations.

The figure shows three methods of transfer of genetic material: (1) transduction (via bacteriophage), (2) transformation (via free deoxyribonucleic acid (DNA)), and (3) conjugation (via plasmid). The antibiotic-resistant gene becomes incorporated into the chromosome by recombination and/or transposition. Adapted from Alekshun and Levy (2007) [23]

One of the most well-known and frequent methods for bacterial populations to transmit genes and enrich themselves with new traits or characteristics is known as horizontal gene transfer (HGT) Khan & Rao, (2019) [24]. HGT involves the exchange of genes between species that do not have a parent-child relationship (Soucy et al., 2015) [25]. Both positive and negative effects may result from this genetic material interchange. A recent review by Emamalipour et al. (2020) [26] stated that HGT has a role in the formation of pathological conditions in cases of

disease, despite the fact that HGT is vital for biodiversity, innovations, and evolution (Jain et al., 2003[27]; Ochman et al., 2000[28]; Soucy et al., 2015) [25]. (Emamalipour et al., 2020) [26].

It occurs through the processes of conjugation (via plasmid and conjugative transposons), transduction (via bacteriophages), or transformation (via incorporation into the chromosome of chromosomal DNA, plasmid, and other naked DNA). This is the main mechanism for the spread of antibiotic resistance in bacteria (Levy & Marshall, 2004) [23]. Additionally, it is essential for the emergence of antibiotic-resistant microbes and the spread of virulence genes. Another uncommon method of gene transfer that was first identified in the 1970s (Solioz & Marrs, 1977) [29] is the use of gene transfer agents. It combines natural transformation with bacteriophage transduction (Lang et al., 2012) [30]. They are tiny, virus-like particles that transmit their whole genome from host cells to other cells (Solioz & Marrs, 1977) [29].

It has long been known that there are bacteria in the environment that are resistant to antibiotics. For instance, there is proof of their existence in caves dating back up to 4 million years (Bhullar et al., 2012) [31]. In addition to being discovered in 30,000-year-old permafrost, antibiotic-resistant bacteria have also been found in the gastrointestinal tracts of Amazon tribe members who have never been exposed to antibiotics (Finley et al., 2013) [32]. (Gibbons, 2015) [33]. Although some bacteria are commensals and naturally colonize individuals, they can cause disease if they spread from their natural habitats (such as the skin and gastrointestinal system) to parts of the body they shouldn't be in (the bloodstream, organs, etc.). Animal manure is how drug-resistant microbes and antibiotics found in animal feed enter the environment (Berendsen et al., 2015[34]; Wichmann et al., 2014) [35]. The proximity of the resistant organisms to one another in the soil encourages horizontal gene transfer through the exchange of genetic determinants (Christensen et al., 1998) [36]. The use of antibiotics in animals and the emergence of antibiotic resistance in people are categorically related in the case of humans (O'Neill, 2016)[37].

Through a variety of channels, including medical facilities, human or animal waste, the use of antibacterial goods, and antibiotic-fortified food and feed supplied to animals, antibiotics are discharged into the environment. Wastewater systems, pharmaceutical manufacturing facilities, food and animal production facilities in agriculture and aquaculture, as well as clinical settings like hospitals, are hotspots for antibiotic-resistant bacteria (Berendonk et al., 2015) [38].

Chapter 3

Methodology

3.1 Data retrieval and annotation

Here, six strains were selected, three Gram-negative (*Escherichia coli, Pseudomonas aeruginosa,* and *Salmonella enterica*) and three Gram-positive (*Mycobacterium tuberculosis, Streptococcus pneumoniae,* and *Staphylococcus aureus*). The PATRIC database was used to extract metadata for finding the strain name, genome ID, genome status, isolation country, hostname, isolation year, and assembly accession number of these six strains. The whole table was downloaded from this very site's metadata.

PATRIC (Pathosystems Resource Integration Center) is a site where all the data about an uploaded bacterial sequence are organized in tables. It is a website related to bacterial bioinformatics from the Bioinformatics Resource Center. This is an information system blending databases with various types of data about bacterial pathogens (transcriptomic, structure, proteomic, and biochemical) along with tools for analysis. Easily accessible, a support system for the biomedical research community's work on bacterial infectious diseases through these integrations of pathogen information. It harmoniously annotates all sequenced bacterial species from GenBank for free within a day using the RAST annotation service. A completely automated service called (Rapid Annotation using Subsystem Technology) is available for annotating bacterial and archaeal genomes. It provides comprehensive phylogenetic tree-wide high-quality genomic annotations for these genomes.

The Linux terminal aided the process of arranging the extracted data under the conda management system. The arrangement was done by the species name and their collection year

from the year 2000 to 2020 and then by their geographic location. A text-based interface called the Linux terminal is used to manage Linux computers. It is merely one of the many tools accessible to Linux users for carrying out any given activity, but it is often regarded as the most effective approach. It's undoubtedly the most direct approach there is, barring the creation of code. It's become so well-liked that Microsoft created PowerShell, its very own open source command line, while Apple switched to using Unix as its foundation and acquired access to the Bash and Z shells.

Consequently, the accession number found in the formed list was used to find and download the whole sequenced genome from the NCBI database via ABRicate and Genomics tools. These are tools to screen the contigs for antimicrobial genes from the sequences found from NCBI. These work like AMRFinderPlus where they identify AMR genes from nucleotide sequences.

Lastly, the AMR genes found were counted and the average was presented as a graph.

Chapter 4

Results

4.1 Average AMR gene count for the past 21 years

The data found are given below

4.1.1 Gram Negative Bacteria

Escherichia coli

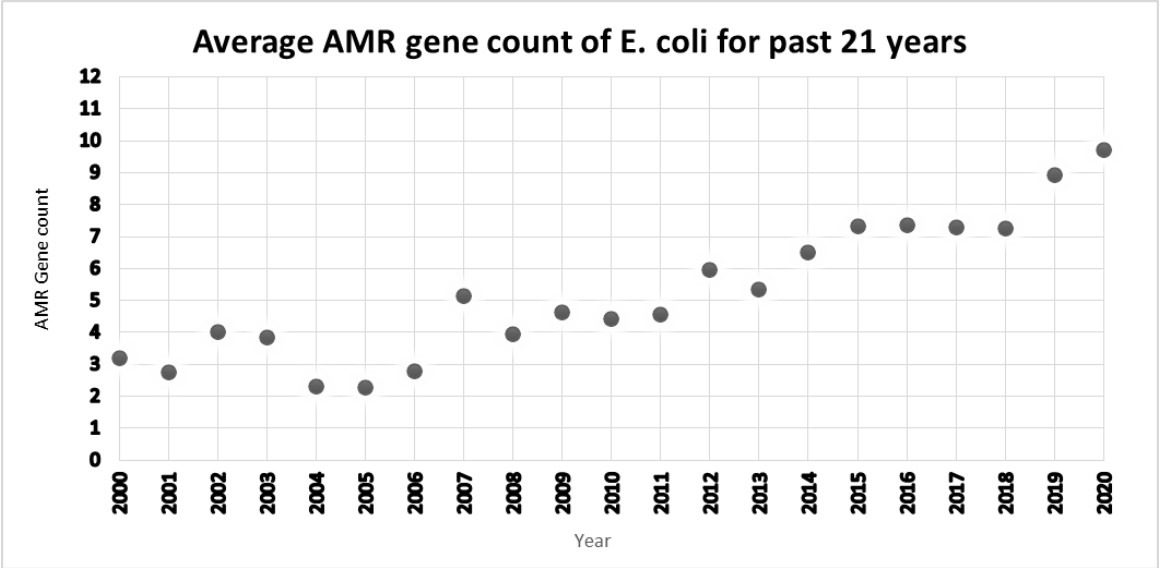

Figure 10: Average AMR gene count of E.coli for the past 21 years

It can be seen that the average gene count which was just above 3 per 100 sequences in 2000 has become more than 10 in 2020. A gradual increase could be seen resulting in more than 3 fold augmentation of the AMR gene count in per 100 sequences.

Salmonella enterica

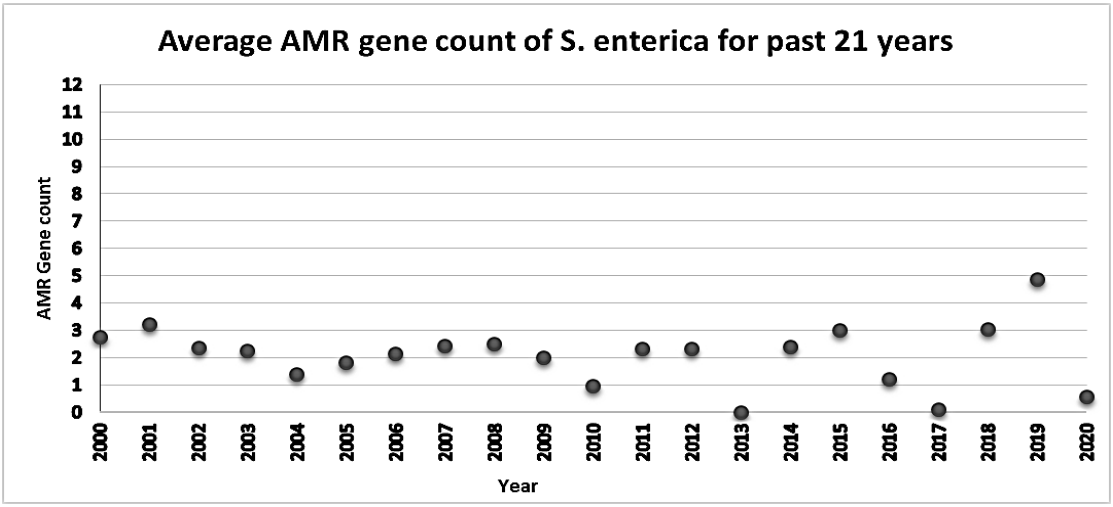

Figure 11: Average AMR gene count of S. enterica for the past 21 years

The average gene count which was around 2 per 100 sequences in 2000 is still around 1 in 2020. No particular change could be seen and can be interpreted as no acquirement of any antimicrobial genes over the years.

Pseudomonas aeruginosa

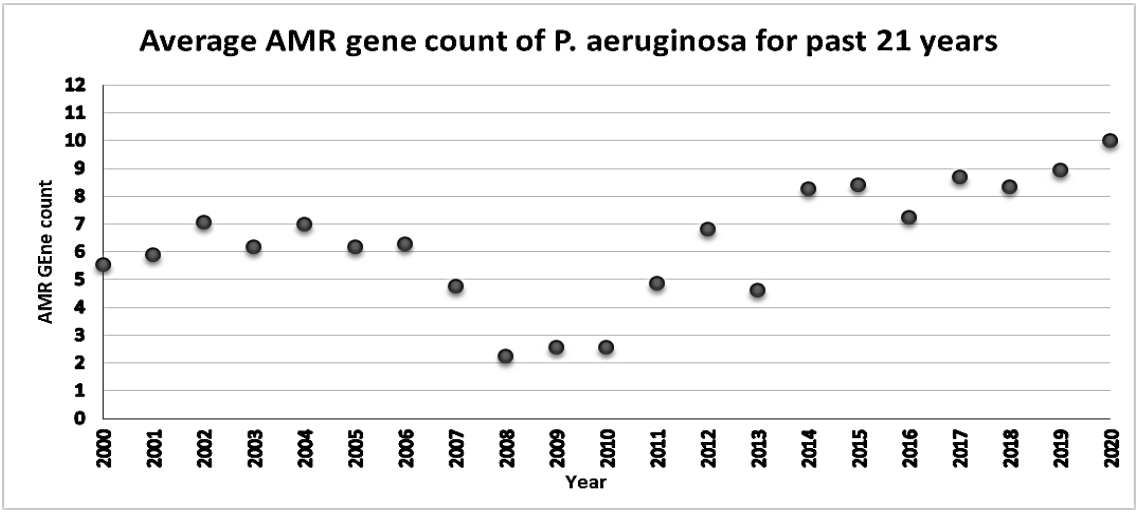

Figure 12:Average AMR gene count of P. aeruginosa for the past 21 years

It shows that the average gene count of just above 5 per 100 sequences in 2000 has become more than 10 in 2020. A gradual increase could be seen resulting in a doubling of the AMR gene count in per 100 sequences.

4.1.2 Gram-Positive Bacteria

Staphylococcus aureus

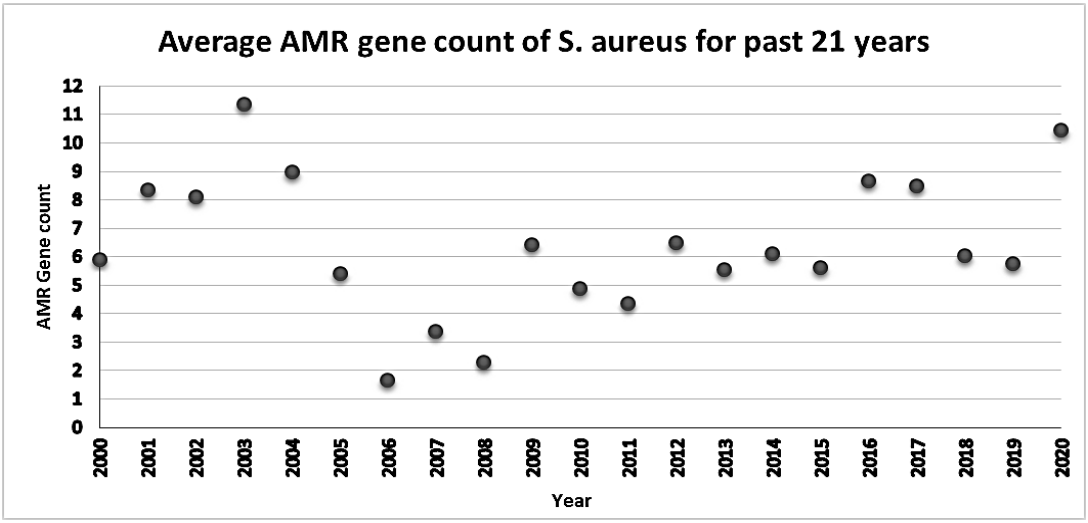

Figure 13: Average AMR gene count of S. aureus for the past 21 years

The graph indicates that the average gene count of just above 5 per 100 sequences in 2000 has become more than 10 in 2020. It increased to almost 12 in the first 4 years and dropped in the next three years. Later raised to 10 till the year 2000.

Streptococcus pneumoniae

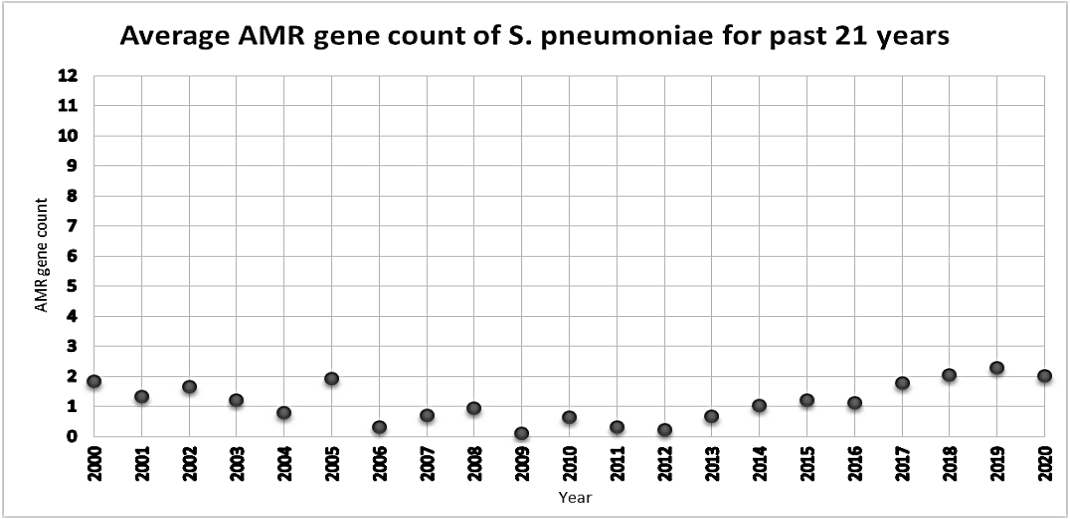

Figure 14: Average AMR gene count of S. pneumoniae for the past 21 years

The figure specifies that the average gene count which was just above 3 per 100 sequences in 2000 has become more than 10 in 2020. A gradual increase could be seen.

Mycobacterium tuberculosis

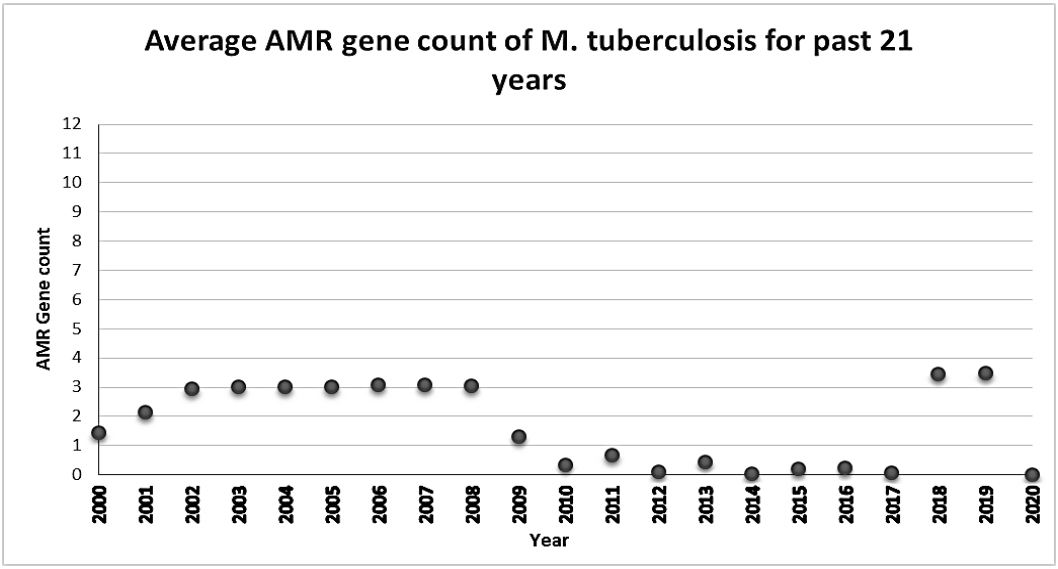

Figure 15: Average AMR gene count of M. tuberculosis for the past 21 years

No particular change or significant fluctuation can be seen from the graph above. The AMR gene count on average stayed from 0-3 throughout the past years.

4.1.3 Temporal trend in AMR

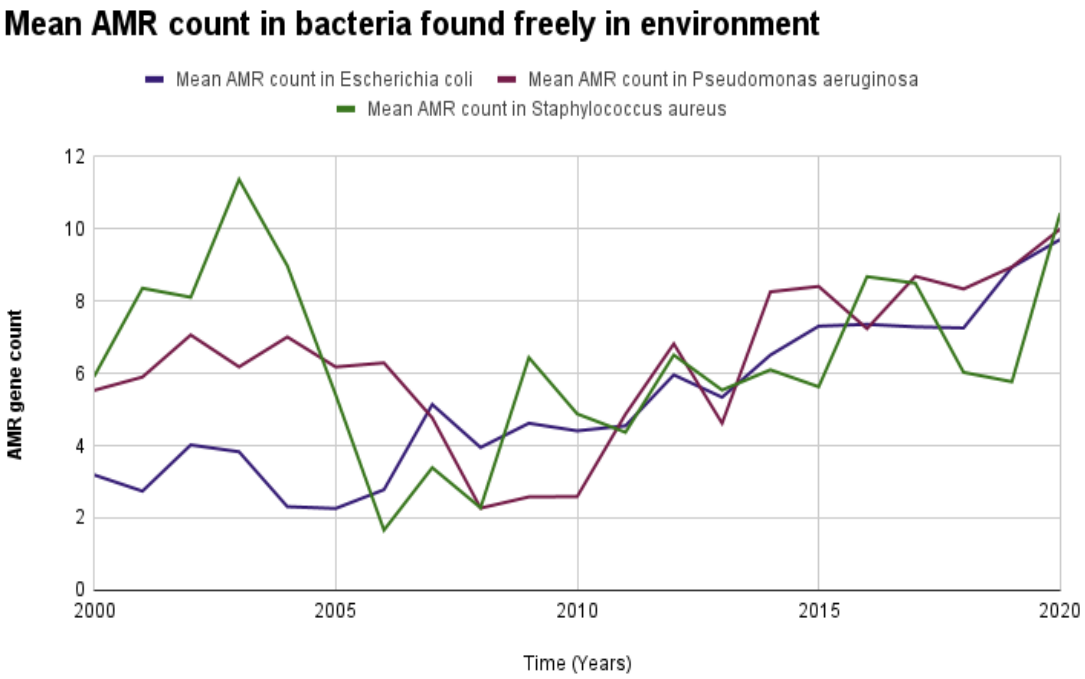

Figure 16: Mean AMR count in bacteria found freely in environment

The graph has shown that a gradual increase in AMR gene count for the three bacteria mostly prevalence in open environment such as rivers, soil, sewage systems. *E. coli*, *P. aeruginosa,* and *S. aureus* rose until almost doubled indicating and influx in antimicrobial gene inside of the DNA of these three bacteria.

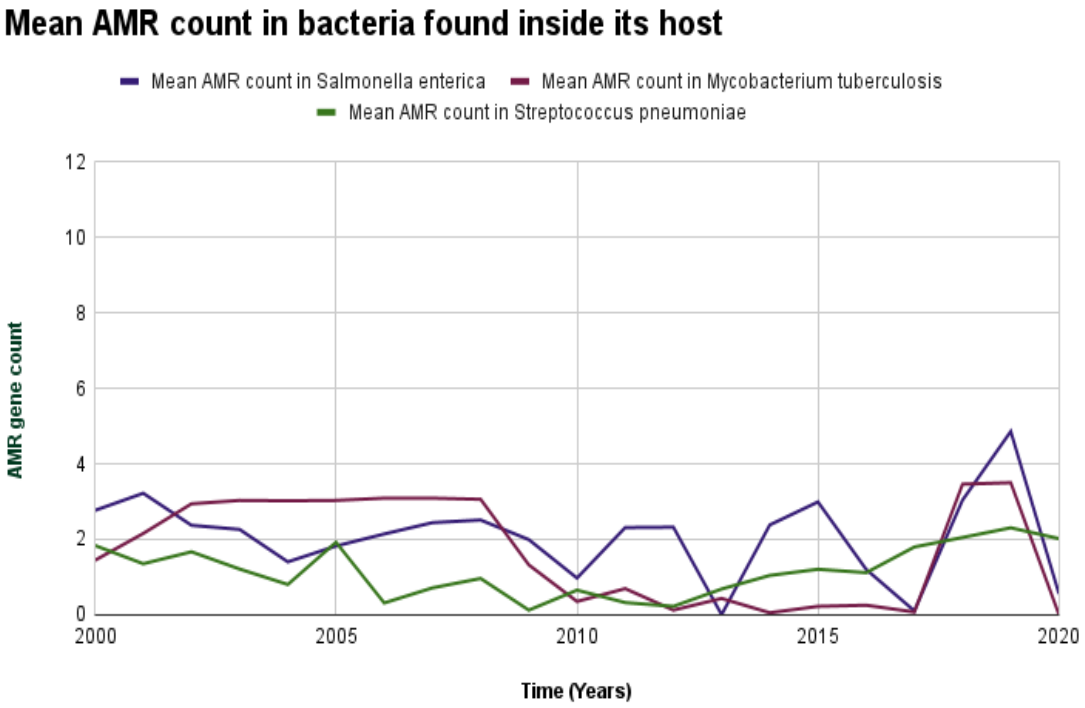

Figure 17: Mean AMR count in bacteria found inside its host

A consistency can be seen in the average AMR gene count when *S. enterica*, *M. tuberculosis*, and *S. pneumoniae* were graphed together. These are the bacteria mostly found inside human/ animal lungs and gut. Here, the values are seen to be stuck below 4 for the past 21 years.

4.2 Average AMR gene count in geographic bias

The data found are given below

4.2.1 Gram Negative Bacteria for both developed and developing countries

Escherichia coli

It can be seen that the average gene count which was just 2 per 500 sequences before 2000 has become only 5 in 2020. A gradual increase could be seen which was not much in case of developed countries for the AMR gene count in per 500 sequences. In contrast, developing

countries' data shows rise from just above 3 to near about 11. Comparing both developed and developing countries' data, it can be seen that for each time range from 1996-2020, the developing countries AMR gene count was somewhat double than that of developed countries.

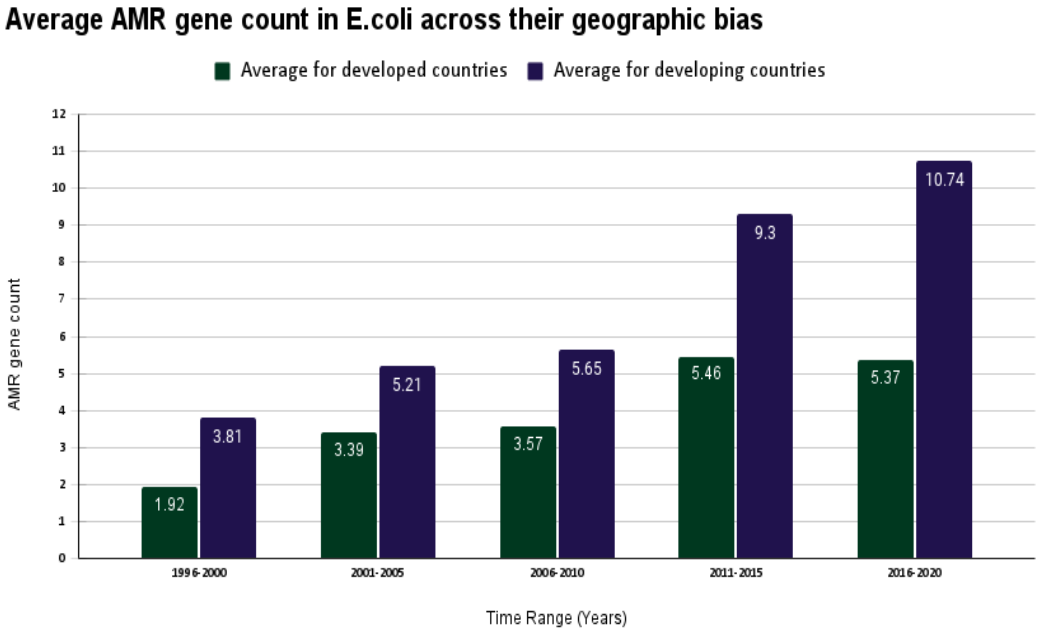

Figure 18: Average AMR gene count of E.coli for its geographic bias

Salmonella enterica

It can be seen that the average gene count which was 3.87 per 500 sequences before 2000 has lowered to only 2.66 in 2020. A fluctuation could be seen for developed countries for the AMR gene count in per 500 sequences. In contrast, developing countries' data shows rise from just above 0.42 to near about 3.61. Comparing both developed and developing countries' data, it can be seen that for each time range from 1996-2020, the developing countries AMR gene count spiked whereas for developed countries, it has lowered.

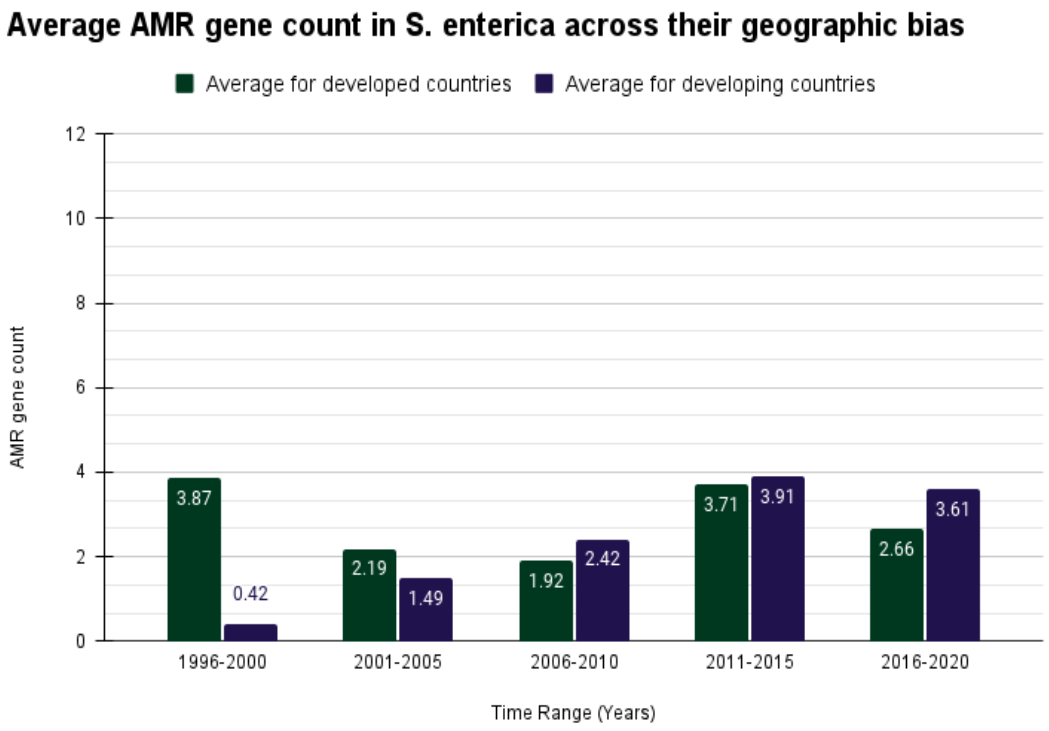

Figure 19: Average AMR gene count of S. enterica for its geographic bias

Pseudomonas aeruginosa

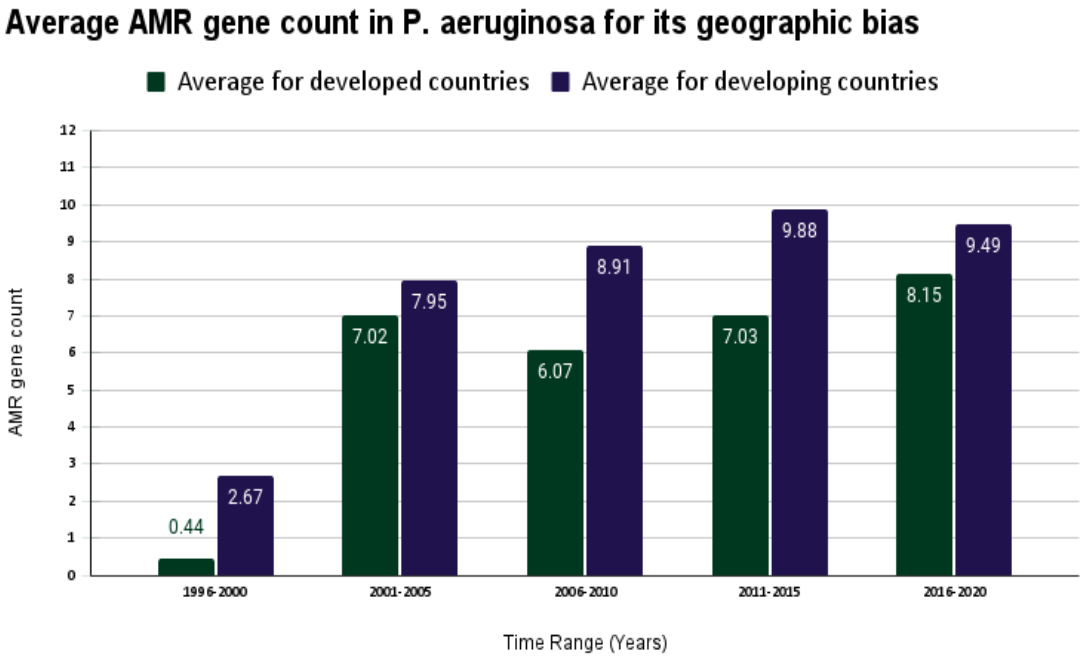

Figure 20: Average AMR gene count of P. aeruginosa for its geographic bias.

It can be seen that the average gene count which was 0.44 per 500 sequences before 2000 has risen to 8.15 in 2020. After the spike for the second time range a little fluctuation could be seen for developed countries for the AMR gene count in per 500 sequences. In contrast, developing countries' data shows rise from 2.67 to 9.49, tripling over the years. Comparing both developed and developing countries' data, it can be seen that for each time range from 1996-2020, the developing countries AMR gene count was more whereas for developed countries, was lower.

4.2.2 Gram-Positive Bacteria for both developed and developing countries

Staphylococcus aureus

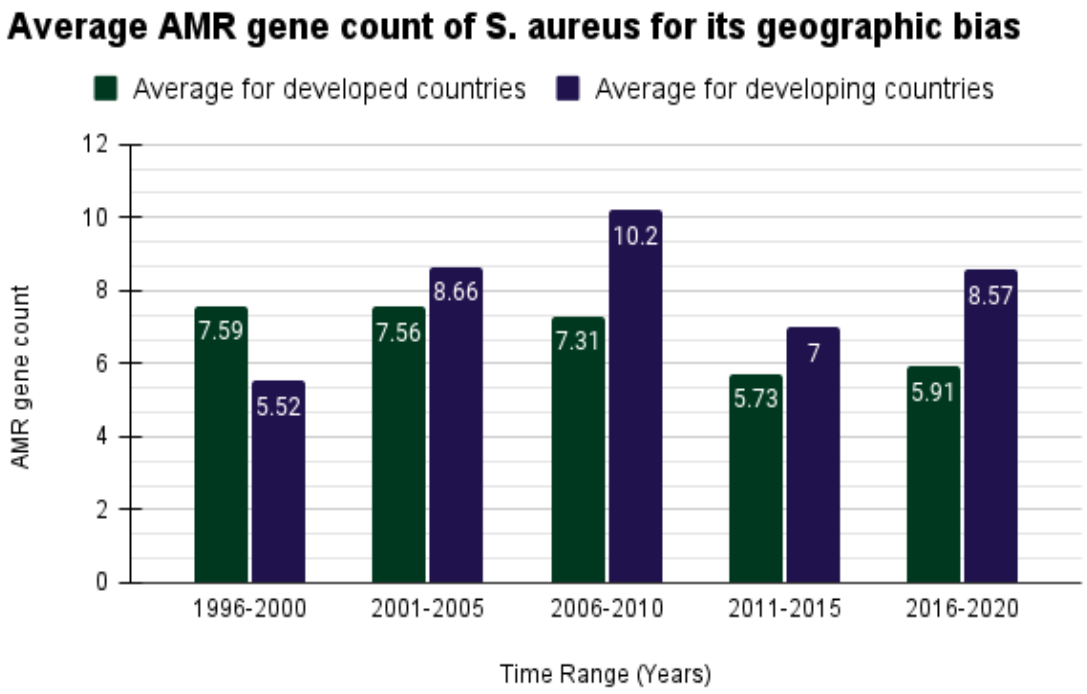

Figure 21: Average AMR gene count of S. aureus for its geographic bias

It can be seen that the average gene count which was 7.59 per 500 sequences before 2000 has fallen to 5.91 in 2020. After an increase till 2010 it lowered and rose again till date for developing countries for the AMR gene count in per 500 sequences. In contrast, developed

countries' data shows fall over the years. Comparing both developed and developing countries' data, it can be seen that for each time range from 2001-2020, the developing countries AMR gene count was more whereas for developed countries, was lower.

Streptococcus pneumoniae

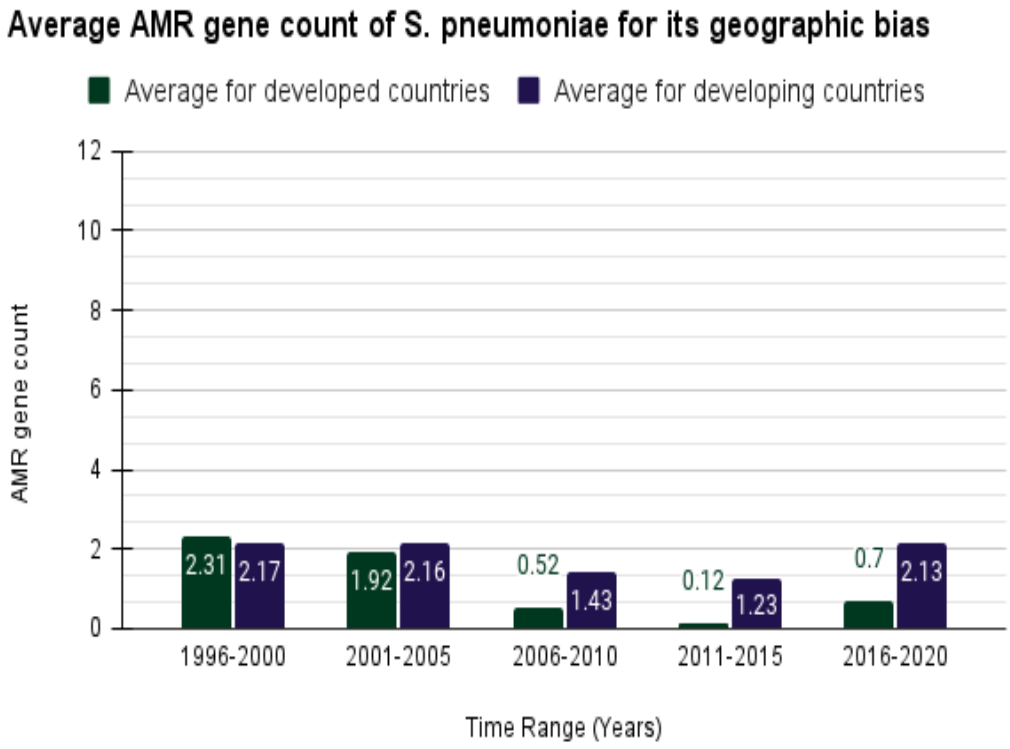

Figure 22: Average AMR gene count of S. pneumoniae for its geographic bias

It can be seen that the average gene count which was 2.31 per 500 sequences before 2000 has fallen to 0.7 in 2020. After a fall till 2015 it doubled in the last 5 years for developing countries for the AMR gene count in per 500 sequences. In contrast, developed countries' data shows fall over the years. Comparing both developed and developing countries' data, it can be seen that for each time range from 2001-2020, the developing countries AMR gene count was more whereas for developed countries, was lower.

Mycobacterium tuberculosis

For both the AMR gene count data per 500 sequences, it can be seen that no to very little fluctuations could be seen for both developed and developing countries.

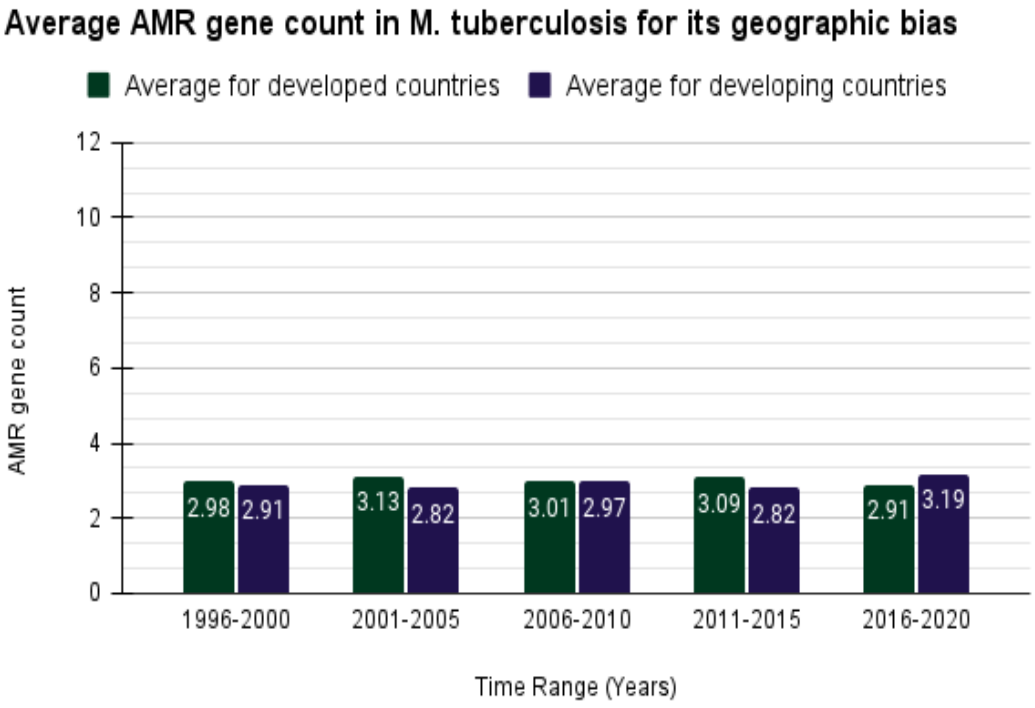

Figure 23: Average AMR gene count of M. tuberculosis for its geographic bias

4.2.3 Spatial trend in AMR

The graph demonstrates that for the three bacteria mostly prevalence in open environment such as rivers, soil, sewage systems. *E. coli*, *P. aeruginosa*, and *S. aureus* are the bacteria whose AMR gene count has risen close to 12 for developing and around 8 for developed. This data points that developing countries yearly data presents more rise than that in case of developed countries.

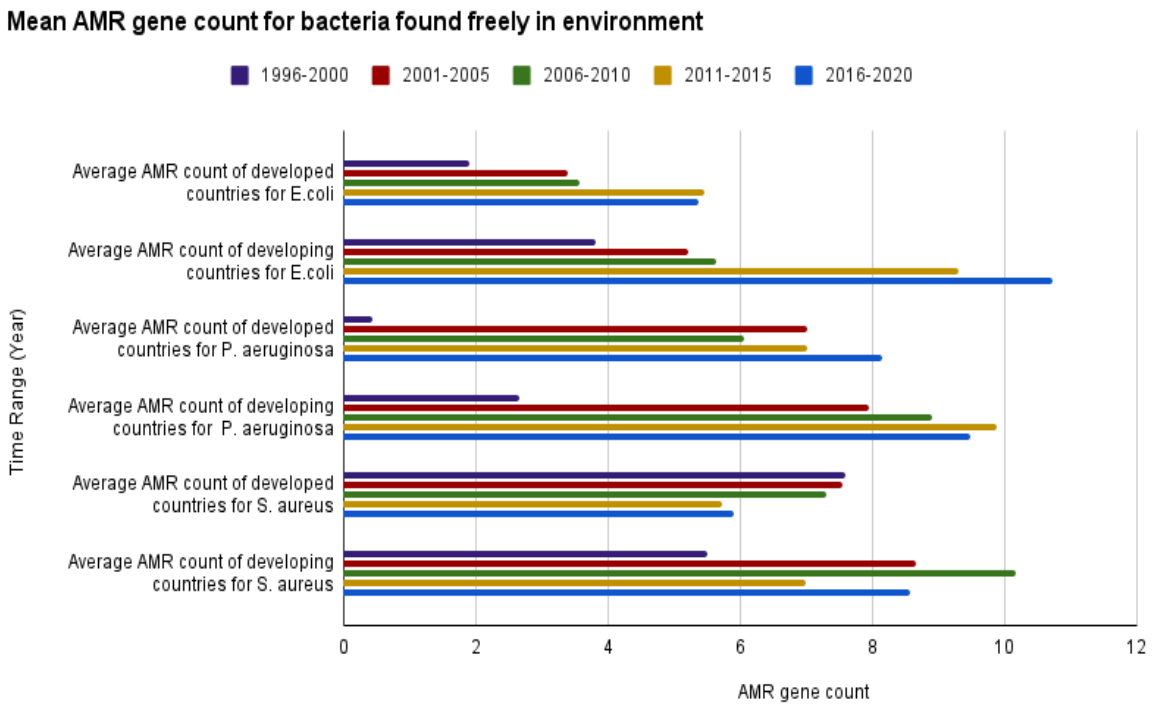

Figure 24: AMR count for spatial data of the bacteria most prevalent in environment.

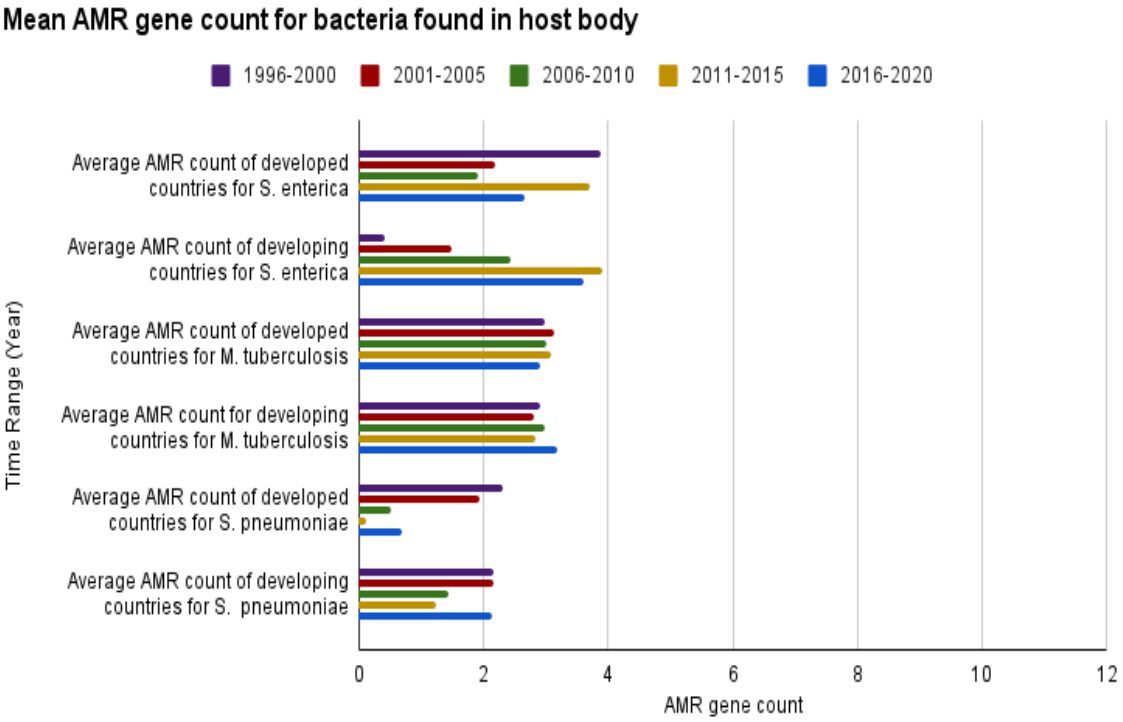

Figure 25: AMR count for spatial data of the bacteria most prevalent in host body.

A consistency can be seen in the average AMR gene count when *S. enterica*, *M. tuberculosis*, and *S. pneumoniae* were graphed together. These are the bacteria mostly found inside human/ animal lungs and gut. Here, the values are seen to be stuck below 4 for both developed and developing countries lowering yearly.

Chapter 5

Discussion

When these bacteria are divided according to their result for Gram staining, two Gram-negative bacteria *E. coli* and *P. aeruginosa* have shown a significant rise in their mean AMR gene count/ 100 sequences each year. *S. enterica* had little fluctuation ending with a lower AMR gene count. Gram-positive on the other hand has shown little fluctuation for the past 21 years in the case of *S. pneumoniae* and *M. tuberculosis*. *S. aureus* was the only Gram-positive bacteria that spiked to almost 12 on average in 2003 and then lowered and rose again till that in 2020. Thus, we can see that dividing these 6 species according to Gram staining did not give any homogenized result.

Temporal data of the past 21 years have shown augmentation of AMR gene count per 100 sequences of more than 2-3 folds for the bacteria found freely in the environment. The increase was seen highest for *E. coli* (tripled), a fecal coliform found in abundance in untreated water. *P. aeruginosa* and *S. aureus* found in soil, and untreated water from hospitals also had a great influx of AMR genes in their genomes. In contrast, bacteria selected to be found enclosed in the host lungs, and gut did not have any significant change in their average AMR gene count ranging between 0-4. This implements that bacteria that are found to be abundant in an open environment like rivers, lakes, and soil tend to acquire more antimicrobial genes than those enclosed in human/ animal bodies. This implements those bacteria being around free DNA, bacteriophage and many other bacteria acquire resistance more than ones enclosed safely in lesser DNA inside the host body. This acquirement of the AMR gene can be through HGT in the case of the bacteria in an open environment. Consequently, by stopping antibiotic abuse, only taking antibiotics when absolutely in need, and maintaining proper hygiene and water

sanitation, these free DNA and bacteria containing AMR genes in soil and water can be reduced, reducing this alarming result.

Worldwide data found have shown that antimicrobial resistance has increased more in developing countries than in developed countries for every 500 sequences. For *E. coli*, the average AMR gene count in developing countries was almost twice that of the developed countries for all time ranges whereas for *P. aeruginosa* and *S. aureus* the data for developing countries showed somewhat higher than that of developed countries. This shows that developed countries where accessibility of the unprescribed antibiotic is rare and sanitation/ hygiene is well maintained had lower AMR gene prevalence in comparison to developing countries. However, for *M. tuberculosis*, *S. enterica*, and *S. pneumoniae* the fluctuation in the gene numbers for developing and developed count was not significant. This proves that a bacterium does not have a huge amount of AMR genes around as free DNA, plasmids in bacteria or AMR in the genome of bacteriophage tend to acquire fewer AMR genes irrespective of its geographic bias.

Chapter 6

Conclusion

To wrap up, we can say that the bacteria most prevalent in open environment acquire more resistance in the course of time rather than the bacteria that stay back in host body. Both Gram-positive and Gram-negative bacteria show this trend. In case of these bacterial AMR gene count across its geographic location and socio-economic status, the countries selected as developed/ richer had a lower AMR gene count in comparison to that of the countries considered as developing/ poorer. This implements that bacteria which is most prevalent in outside environment tend to acquire more resistant and improving sanitization and reducing drug use can ameliorate our health.

References

1. Aminov R. I. (2010). A brief history of the antibiotic era: lessons learned and challenges for the future. *Frontiers in microbiology*, *1*, 134.
2. Aminov, R.I. (2009), The role of antibiotics and antibiotic resistance in nature. Environmental Microbiology, 11: 2970-2988
3. Society, M. (n.d.). *The history of antibiotics*. Microbiology Society. Retrieved August 30, 2022, from https://microbiologysociety.org/members-outreach-resources/outreach-resources/antibiotics-unearthed/antibiotics-and-antibiotic-resistance/the-history-of-antibiotics.html
4. Britannica, T. Editors of Encyclopedia (2022, August 29). *antibiotic*. *Encyclopedia Britannica*.
5. Sandle, T. 'Gram's Stain: History and Explanation of the Fundamental. Technique of Determinative Bacteriology', IST Science and Technology Journal, April 2004 (No. 54), pp3-4
6. Lim, J. Y., Yoon, J., & Hovde, C. J. (2010). A brief overview of Escherichia coli O157:H7 and its plasmid O157. *Journal of microbiology and biotechnology*, *20*(1), 5–14.
7. Anastasi, E. M., Matthews, B., Stratton, H. M., & Katouli, M. (2012). Pathogenic Escherichia coli found in sewage treatment plants and environmental waters. *Applied and environmental microbiology*, *78*(16), 5536–5541.
8. Diggle, S. P., & Whiteley, M. (2020). Microbe Profile: *Pseudomonas aeruginosa*: opportunistic pathogen and lab rat. *Microbiology (Reading, England)*, *166*(1), 30–33.
9. Taylor TA, Unakal CG. Staphylococcus Aureus. [Updated 2022 Jul 18]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK441868/
10. Aydin A, Sudagidan M, Muratoglu K (2011) Prevalence of staphylococcal enterotoxins, toxin genes and genetic relatedness of foodborne Staphylococcus aureus strains isolated in the Marmara region of Turkey. International Journal of Food Microbiology 148:99–106
11. Tong, S. Y., Davis, J. S., Eichenberger, E., Holland, T. L., & Fowler, V. G., Jr (2015). Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clinical microbiology reviews*, *28*(3), 603–661
12. Wendy J. et. al, (2015). Biology and Diseases of Ruminants (Sheep, Goats, and Cattle). Laboratory Animal Medicine. American College of Laboratory Animal Medicine. 623-694 (3).
13. Arora, D., Kumar, S., Jindal, N., Narang, G., Kapoor, P. K., & Mahajan, N. K. (2015). Prevalence and epidemiology of Salmonella enterica serovar Gallinarum from poultry in some parts of Haryana, India. Veterinary world, 8(11), 1300–1304. https://doi.org/10.14202/vetworld.2015.1300-1304
14. Tuberculosis. Broiler Health. Poultry Production Manual. ANIMAL & FOOD SCIENCES. retrieved from http://afs.ca.uky.edu/poultry/chapter-4-tuberculosis
15. Nikaido H. (2009). Multidrug resistance in bacteria. Annual review of biochemistry, 78, 119–146. https://doi.org/10.1146/annurev.biochem.78.082907.145923
16. Alekshun, M. N., & Levy, S. B. (2007). Molecular mechanisms of antibacterial multidrug resistance. Cell, 128, 1037–1050.
17. Ayukekbong, J. A., Ntemgwa, M., & Atabe, A. N. (2017). The threat of antimicrobial resistance in developing countries: Causes and control strategies. Antimicrobial Resistance & Infection Control, 6, 47.

18. Choudhury, R., Panda, S., & Singh, D. (2012). Emergence and dissemination of antibiotic resistance: A global problem. Indian Journal of Medical Microbiology, 30, 384.
19. Colodner, R., Rock, W., Chazan, B., Keller, N., Guy, N., Sakran, W., & Raz, R. (2004). Risk factors for the development of extended-spectrum beta-lactamase-producing bacteria in nonhospitalized patients. European Journal of Clinical Microbiology and Infectious Diseases, 23, 163–167.
20. Gashaw, M., Berhane, M., Bekele, S., Kibru, G., Teshager, L., Yilma, Y., Ahmed, Y., Fentahun, N., Assefa, H., & Wieser, A. (2018). Emergence of high drug resistant bacterial isolates from patients with health care associated infections at Jimma University medical center: A cross sectional study. Antimicrobial Resistance & Infection Control, 7, 138.
21. Nikaido, H. (2009). Multidrug resistance in bacteria. Annual Review of Biochemistry, 78, 119–146.
22. Tanwar, J., Das, S., Fatima, Z., & Hameed, S. (2014). Multidrug resistance: An emerging crisis. Interdisciplinary Perspectives on Infectious Diseases, 2014,541340.
23. Alekshun, M. N., & Levy, S. B. (2007). Molecular mechanisms of antibacterial multidrug resistance. Cell, 128, 1037–1050.
24. Khan, A., & Rao, T. S. (2019). Molecular evolution of xenobiotic degrading genes and mobile DNA elements in soil bacteria. In Surajit Das & Hirak Dash Microbial diversity in the genomic era (pp. 657–678). Elsevier.
25. Soucy, S. M., Huang, J., & Gogarten, J. P. (2015). Horizontal gene transfer: Building the web of life. Nature Reviews Genetics, 16, 472–482.
26. Emamalipour, M., Seidi, K., Zununi Vahed, S., Jahanban-Esfahlan, A., Jaymand, M., Majdi, H., Amoozgar, Z., Chitkushev, L. T., Javaheri, T., Jahanban-Esfahlan, R., & Zare, P. (2020). Horizontal gene transfer: From evolutionary flexibility to disease progression. Frontiers in Cell and Developmental Biology, 8,1–16.
27. Jain, R., Rivera, M. C., Moore, J. E., & Lake, J. A. (2003). Horizontal gene transfer accelerates genome innovation and evolution. Molecular Biology and Evolution, 20, 1598–1602.
28. Ochman, H., Lawrence, J. G., & Groisman, E. A. (2000). Lateral gene transfer and the nature of bacterial innovation. Nature, 405, 299–304.
29. Solioz, M., & Marrs, B. (1977). The gene transfer agent of Rhodopseudomonas capsulata: Purification and characterization of its nucleic acid. Archives of Biochemistry and Biophysics, 181, 300–307.
30. Lang, A. S., Zhaxybayeva, O., & Beatty, J. T. (2012). Gene transfer agents: Phage-like elements of genetic exchange. Nature Reviews Microbiology, 10, 472–482.
31. Bhullar, K., Waglechner, N., Pawlowski, A., Koteva, K., Banks, E. D., Johnston, M. D., Barton, H. A., & Wright, G. D. (2012). Antibiotic resistance is prevalent in an isolated cave microbiome. PLoS One, 7, e34953.
32. Finley, R. L., Collignon, P., Larsson, D. J., McEwen, S. A., Li, X.-Z., Gaze, W. H., Reid-Smith, R., Timinouni, M., Graham, D. W., & Topp, E. (2013). The scourge of antibiotic resistance: the important role of the environment. Clinical Infectious Diseases, 57, 704–710.
33. Gibbons, A. (2015). Resistance to antibiotics found in isolated Amazonian tribe. Science. https://doi.org/10.1126/science.aab2509
34. Berendsen, B. J., Wegh, R. S., Memelink, J., Zuidema, T., & Stolker, L. A. (2015). The analysis of animal faeces as a tool to monitor antibiotic usage. Talanta, 132, 258–268.
35. Wichmann, F., Udikovic-Kolic, N., Andrew, S., & Handelsman, J. (2014). Diverse antibiotic resistance genes in dairy cow manure. mBio, 5,e01017-13.

36. Christensen, B. B., Sternberg, C., Andersen, J. B., Eberl, L., Møller, S., Givskov, M., & Molin, S. (1998). Establishment of new genetic traits in a microbial biofilm community. Applied and Environment Microbiology, 64, 2247–2255.
37. O'Neill, J. (2016). Tackling drug-resistant infections globally: Final report and recommendations.
38. Berendonk, T. U., Manaia, C. M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., & Pons, M.-N. (2015). Tackling antibiotic resistance: The environmental framework. Nature Reviews Microbiology, 13, 310.

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