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Molecular Epidemiology and Prevalence of Antibiotics Resistance Genes in Smog Particulate Matter

by

Muhammad Zameer

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Dedicated to ALLAH Almighty, Hazrat Muhammad (PBUH) and my Mother Janat Bibi who has been passed away from us due to Covid-19 durring the completion of my thesis.

CERTIFICATE OF APPROVAL

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Muhammad Zameer

(MBS191026)

THESIS EXAMINING COMMITTEE

S. No.	Examiner	Name	Organization
(a)	External Examiner	Dr Arshad Islam	NUST, Islamabad
(b)	Internal Examiner	Dr. Syeda Marriam Bakhtiar	CUST, Islamabad
(c)	Supervisor	Dr. Arshia Ameen But	CUST, Islamabad

Dr.Arshia Ameen But
Thesis Supervisor
April, 2021

Dr. Sahar Fazal
Head
Dept. of Biosciences & Bioinformatics
April 2021

Dr. Muhammad Abdul Qadir
Dean
Faculty of Health & Life Sciences
April 2021

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Abstract

In recent years, we are facing new multiple challenges that are also interconnected with one another. Increasing levels of Smog and dissemination of antibiotics resistance are current challenges which are interlinked. Air particulate matter provides a physical surface to which Antibiotics resistance genes and resistant bacteria can adhere more stably. During the smog, concentration of air particulate matter increase many times, that is why more antibiotics resistance genes and bacteria are present in smog and heavy haze air. Bacteria can acquire antibiotics resistance genes form particulate matter and can transfer to other bacteria through horizontal gene transfer. Due to misuse, large amount of antibiotics are released into natural environments. These antibiotics create a selection pressure on bacterial populations and those bacteria survive which posses ARGs. In this study, we have collected samples of air particulate matter during somg and none smog days from Islamabad and investigated the presence of cultureable antibiotics resistant bacteria and antibiotic resistance genes. Six antibiotics resistance genes blaTEM, tetM, sul3, vanA, qnrA and intl1, were selected in this study to check the dissemination of antibiotics resistance through air particulate matter. After isolating bacterial strains, we monitored the resistant strains by disc diffusion method. We have successfully identified five multidrug resistant bacterial strains by gene sequencing. These strains were *Lactobacillus reuteri, Staphylococcus arlettae, Kocuria sediminis, Limosilactobacillus reuteri* and a member of *Burkholderiales*. We extract DNA from air particulate matter of smog sample and performed PCR for selected genes with previously defined primers and the results were monitored through gel electrophoresis. In our results, we have found the presence of three resistance genes. blaTEM, qnrA, intl1 were present while sul3, tetM and vanA genes were absent. Inhalation of ARGs with air particulate matter during smog can create serious threats of dissemination of antibiotics resistance genes in human microbiota, especially when a person is going through antibiotic therapy. Other consequences of inhalation of air particulate matter are still need to be investigated.

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Abbreviations

AMP	Ampiciline
ARGs	Antibiotics Resistance Genes
ARBs	Antibiotics Resistance Bacteria
CTAB	Cetyltrimethylammonium Bromide
CRO	Ceftriaxone
EDTA	Ethylenediaminetetraacetic Acid
GNB	Gram Negative Bacteria
IMP	Imipinum
IZD	Inhibition Zone Diameters
MDR	Multi-Drug Resistance
MRSA	Methicillin-Resistant *Staphylococcus aureus.*
NAL	NalIdiXIc Acid
PM	Particulate Matter
SDS	Sodium Dodecyl Sulfate.
TSA	Trypticase Soy Agar or Tryptone Soya Agar
TET	Tetracycline
VAN	Vancomycine

Chapter 1

Introduction

1.1 Smog

In many developing countries, heavy air pollution is a serious problem and it is resulting in the formation of smog [1]. Population explosion and industrialization has worsened the air quality of the urban cities. In a report of World Health Organization, it was declared that air pollution is the world's largest environmental health risk. In South Asia, Pakistan is a country with most of its people live in cities [2]. In the air, when smoke and fog mixes together a new kind of air pollution is formed that is termed as smog. Two factors are important in the formation of smog: (1) low water vapors in the clouds so that these water vapors are unable to convert themselves in rain droplets and (2) decreased wind speed. Smog is a big problem all over the world; especially urban cities are facing severe conditions of smog in fewer months of the year, which is hazardous for the people living to these cities. Chemicals in the smog like sulphar dioxide, ground level ozone, nitrogen dioxide and carbon monoxide are very harmful especially for senior citizens those having heart and lung diseases such as emphysema and asthma. Lahore the capital of Punjab is second most populated city in the country and ranked first in air pollution. The Rights group, a U.K based organization has warned recently that the air quality of Pakistan is so poor that it can harm the health of hundreds of

thousands of people. Similarly Faisalabad, is the industrial hub of the country, have worse conditions of smog. Whole the southern Punjab and some parts of Sindh experiences heavy smog during few months of the year. Smog conditions are also worsens time to time in Islamabad, the capital of the country. In fact smog has become a fifth season of the country.

Islamabad is one of the most over populated cities in Pakistan. The population of Islamabad is over than 1 million. The average level of pollution in the city with respect to PM 2.5 is 35.2 g/m^3, in year 2019. It means the quality of in Islamabad is not safe, and can cause a large number of health related problems for its citizens, especially for senior citizens those having heart and lung diseases such as emphysema and asthma and all other vulnerable demographics like children, and people with weaker immune systems. The main reason of pollution in the city includes rapid urbanization and some general habits that add to air contamination such as burning of waste and biomass for cooking of food and heating purpose. Apart from these reasons, there are some other factors which also contributing in air pollution like factory emission from scraping materials, bricks production units and chemical production plants. Lastly, the most prominent source of air pollution in Islamabad is emissions from vehicles, together with factory smoke and fumes, making up the most of pollution levels. The air quality index (AQI) is the index

Smog Conditions in Islamabad

of air quality that ranges from 0 to 500. If the AQI is range from 0 to 50 the air

quality will be considered as good and satisfactory and there is little or no risk of inhalation this air for all people of all age groups. If the AQI is range from 51 to 100 the air quality will be considered as moderate and acceptable, however; there is a mild risk of inhalation this air for those people which are very sensitive to air pollution. If the AQI is range from 101 to 150 the air quality will be considered as unhealthy for sensitive people, however; only the sensitive people feel the health risk by inhaling this air. General public will not be affected.

If the AQI is range from 150 to 200 the air quality will be considered as unhealthy. General public will start affected now and may experience health risks. If the AQI is range from 200 to 250 the air quality will be considered as very unhealthy. General public will be affected now and may experience health risks and heath emergency may arise. If the AQI is range from 251 the air quality will be considered as very hazardous. Every person will be affected and may experience more serious health risks at this level.

From last few years he data of air quality index showed that the worst air pollution and Pm2.5 concentration in Islamabad prevails in the same months of year as it occurs in other parts of our country. The most unpolluted and cleanest months are March to May. The concentration of PM2.5 is 18.6 micro g/m^3 in March which reduces to 17.2 micro g/m^3 April and become minimum in the month of May which is 14.6 microg/m^3. May is the cleanest month of Pakistan in the whole year.

These PM concentrations considered as almost good that means the air of Islamabad in these months is safe for all citizens of all ages. As the month May passes the PM2.5 concentration start rising again. In June the concentration of PM2.5 reaches to 20.5 micro g/m^3. in July its concentration reaches to 31.7 micro g/m^3. PM2.5 concentration continues to rise in next months. November and December are the driest months of the year and the conditions for PM2.5 may raise more than 100 micro g/m^3. This level of PM2.5 concentrations are considered as very unhealthy for the general public.

1.2 Particulate Matter

Particulate matter (PM) is a potent component of air pollution [3] and it has various affects on environmental health and general public health. Chemistry of PM is very complicated. It is a made up of biological components like microbes, organic compounds and inorganic substances [4]. Many studies have indicated that high concentration of PM in air cause air pollution that can reduce visibility and also plays its important role in climatic changes [5-9]. As these ARGs are present on air particulate matter that can travel long distances with wind circulations so the other place where antibiotic resistance is not present can be affected with second hand exposure of ARGs [10]. As the anthropogenic activities are more in urban area, it is expected that more ARGs are loaded on to particulate matter from urban cities. Hence urbanization is involved in another global problem of antibiotics dissemination.

Particulate matter is the sum of all solid and liquid particles suspended in air many of which are hazardous. This complex mixture includes both organic and inorganic particles, such as dust, pollen, soot, smoke, and liquid droplets. These particles vary greatly in size, composition, and origin.

Suspended particulate matter (PM) in the air during the smog season increases and it reaches to our lungs with breathing. Exposure to PM causes increased respiratory and cardiovascular disease [11] and are strongly associated with increased acute respiratory infections, including pneumonia. Many studies have also shown that inhalation of PM causes host tissue damage, inflammation, oxidative stress and lead to alteration in cardiovascular functioning, as well as significantly impacting the immune response by impairing macrophage function [12]. However, these studies do not fully account for the all PM-related diseases in humans. Physical and chemical characteristics of air PM has been explored extensively, however; there is little knowledge is available regarding characterization of antibiotics resistant genes (ARGs) and antibiotics resistant bacteria (ARBs) that are present on the air PM during the smog and non-smog days[13].

1.3 Antibiotic Resistance Genes and Their Prevalence on PM

In past few decades the misuse of antibiotics in clinical practices, veterinary and agriculture, the antibiotics resistance become a serious threat. Most effective drugs against infectious diseases are losing their efficiency. Antibiotics resistance genes (ARGs) and their dissemination is of global concern. Due to presence of ARGs in various environments, it has been recognized that ARGs are potent contaminants of environment [14, 15]. In fact ARGs are also becoming major contaminant of particulate matter which was previously not considered. More importantly the urban air is mostly polluted by ARGs and many cities are facing different health problems that are associated with airborne ARGs. As a potent air pollutant, airborne ARGs when inhaled, they reach to our respiratory track and here they disrupt the equilibrium among the bacterial community. Due to their aerial transport of ARGs, remote regions which are not using antibiotics may have the "second hand" exposure of ARGs. Humans have a constant risk of exposure to these ARGs through inhalation that is why characterization of ARGs in the air particulate matter is necessary. On the other hand multi-drug resistant bacteria have become big challenge in the treatment of infectious diseases i.e.; Methicillin-resistant Staphylococcus aureus MRSA [16]. Presence of such bacteria on air particulate matter is reported in different studies. During the smog season, amount of particulate matter in air increases many folds. In a previous study it was pointed out that these particles provides more adhesion sites on highly polluted days which allow bacterial cells to suspend more stably in the air[17].

1.4 Rationale of The Study

The main goal for this study is to advance the knowledge about airborne antibiotics resistant bacteria and antibiotics resistance genes on air particulate matter during the smog days and their role in dissemination of antibiotics resistance. A better

awareness of the abundance and types of airborne ARGs is critical to understanding current infectious disease transmission and providing valuable information for re-evaluating air quality assessment practice As for as in my knowledge, it is the first research in our country on this topic. It is a pilot study that will open a gate for the researcher to investigate the presence of ARGs and ARBs on PM in different cities across the country during the smog and non smog days. Furthermore, by expanding this work we will be able to identify the sites (urban ruler, agricultural, industrial or clinical) which are considered as hotspot for emission of PM that is loaded with ARGs and ARBs. In a previous study in 2018, a global survey for ARGs in air of big cities was conducted [18]. This research has become journal's most read article of the month. This indicates the importance of this topic. It means characterization of ARGs on air particulate matter is most important and timely subjects of the field and are bound to have significant impact. In this research study we will discuss the potential role of air particulate matter in dissemination of antibiotic resistance.

1.5 Problem Statement

Air-particulate matter serves as solid surface for various airborne bacteria as well as substrate for antibiotic resistance genes. With varying concentration of air particulate matter in smog and non- smog seasons intensity of airborne bacteria may have change considerably. Antibiotic resistance genes on particulate matter are involved in dissemination of antibiotics resistance. The prevalence of ARBs and ARGs on PM during smog need to be studied.

1.6 Aim of Study and Objectives of The Study

This study entails the following aim and objectives:

- To unravel the role of smog in antibiotics resistance dissemination through air particulate matter and its hazardous effects to public health.
- To culture microbial strain from particulate matter present in the smog and non-smog days and isolate resistant bacteria and identify multi-drug resistant bacterial strains.
- To check the presence of clinically important ARGs on particulate matter during smog and non-smog days.
- To find interlink between antibiotics prescription practices and ARGs presence on particulate matter during smog.

Chapter 2

Literature Review

2.1 Antibiotics Resistance

Bacteria have successfully inhabited the planet earth for approximately three and a half billion years that are much longer than human beings. Continuous survival of the bacteria on planet earth is because of their ability to adopt quickly according to their environment. Over their survival journey bacteria have encountered various challenges. Use of antibiotics at large scale in last few decades is one of those challenges that bacteria have faced in recent past. However, bacteria have successfully overcome this challenge and they have developed resistance against these antibiotics. Penicillin was the first antibiotic that was used clinically but penicillin resistant bacteria were soon isolated in 1941. Similarly resistance against sulfonamide was reported in 1939 [19-21]. Streptomycin was initially considered as breakthrough in treatment of tuberculosis [22]. However, resistance against streptomycin was observed soon in *Mycobacterium tuberculosis*, which resulted in relapses [23]. Today, the evolution of microbial pathogens able to resist antibiotics treatments is seen as one of the most pressing public health crises [6-9]. Moreover some bacterial strains are multi-drug resistance. Currently we are facing the threat of bacterial supper bugs, which are making the infectious diseases worst. Recently hyper virulent *Klebsiella pneumoniae* resistant to all antibiotics tested

recently appearing in Chinese hospitals [24]. Above all, the rate at which bacteria developing antibiotic resistance is alarming. According to different studies antibiotic resistance will be leading cause of death in 2050.

2.2 Role of Antibiotics in Natural Environment

Naturally antibiotics are mostly produced by soil microorganisms. The basic role of these antibiotics in natural environment is seem to be provide benefit to the bacteria and help them to compete with other bacteria in the same environment by inhibiting the growth of other bacteria [25]. However; recent study showed that the basic role of antibiotics is not the inhibiting the growth of other bacteria but these antibiotics was involved in signaling processes among the bacterial communities at their low concentrations. At the low concentrations these antibiotics are involved in many transcriptional processes cell to cell interactions among the bacterial cells [26, 27]. These processes are entirely different from those processes which are the part of stress response of bacteria. Many of signaling processes that are initiated by antibiotics are very unique to bacterial communities. As human start production of antibiotics and their extensive misuse results in the change in concentrations of antibiotics in natural environment. In fact misused antibiotics mostly reach to the natural environments where these antibiotics disrupt the signaling interactions. Hence the concentration of these antibiotics becomes so high that their natural role of signaling shifts into killing or inhibiting the growth of other bacteria in the environment.

2.3 Accumulation of Antibiotics in Environment and its Role in Antibiotics Resistance

The demand of antibiotics has been increased enormously since last many years. It is estimated that total demand for antibiotics might had increased 36 percent

from year 2000 to 2010. Today antibiotics are commonly used in the treatment of bacterial infections both in animals and agriculture. Beside their clinical use antibiotics are commonly used in animal forming and fish forming [31]. There is a greater chance that many antibiotics remains unused and escape into the natural environments and accumulate in different water reservoirs like ponds lakes and rivers [32]. According to U.K government the misuse of antibiotics in agriculture and animal husbandry is so common that it accelerated the process of antibiotics resistance so many times. It is now expected that the resistance in bacteria due to misuse of antibiotics may have become the leading cause of death in 2050. Misused antibiotics reach into the water bodies by using different routes. Most common route is the release of untreated waste water from water treatment plants. Many prescribed antibiotics remains unused after the completion of antibiotics treatment. Improper disposal off these antibiotics is another way these antibiotics may release in to natural environment and reach to the water bodies. A large amount of antibiotics is released from agricultural site with runoff water and reaches to the water bodies. It is noted that the concentration of antibiotics in water reservoirs is directly related to treatment of waste water at water treatment plants [33]. Water treatment plants have the capacity to remove solid wastes and

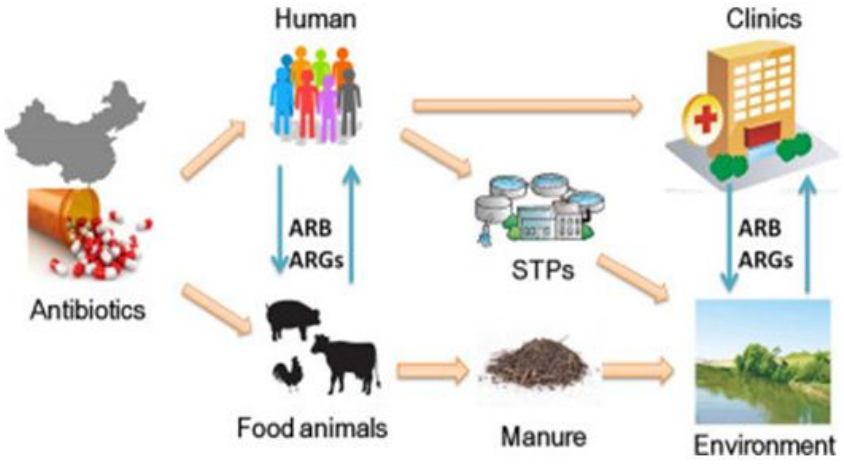


FIGURE 2.1: Routes of accumulation of antibiotics in environment

suspended organic matter from water but these water treatment plants are unable to remove smaller molecules like antibiotics biocides and disinfectants. Antibiotics

that are given to animals may remain unused in the body and excreted into the environment through fecal materials. These fecal materials are used as manure in agricultural lands; hence these antibiotics increase the concentration of antibiotics in agricultural lands. As antibiotics reaches into soil, some of them are inactivated by the soil but many remains active where they creates a higher concentrations of antibiotics which results in antibiotics pressure on natural bacteria of soil. In many natural environments like surface water, industrial effluents and soil the concentration of antibiotics is almost thousand times lower than it is used in antibiotic therapies. However; this minimal concentration of antibiotics is fair enough that it caused a shift in the role of natural antibiotics from signaling to growth inhibition of bacteria.

Concentration of antibiotics in many natural environments like surface water industrial effluents and soil is thousand time lowers as compare to the concentration of antibiotics that is used for bacterial infections in clinical antibiotic therapies. However this minimal concentration of antibiotics is fair enough to change the role of antibiotics from signaling among the bacterial population to growth inhibition. In the presence of these antibiotics bacteria feels a selective pressure of antibiotics and rushes for acquiring antibiotics resistance genes. Beside antibiotics other pollutants like heavy metal I,e; lead, copper, zinc, cadmium, nickel, chromium and mercury are toxic not only for the natural bacteria in the environment but plants and animals are also being affected by these pollutants. Presence of heavy metal along with antibiotics ergs the bacteria to acquire resistance genes through the process of horizontal gene transfer. Hence all these factors accelerated the process of antibiotics resistance. In the presence of stressors, like antibiotics and other environmental pollutants like heavy metals the rate of exchange of genetic material increases between bacterial cells [34]. The selective pressure of these antibiotics even at the sub-inhibitory level is shown to increase the mutation rate and gen recombination frequencies by the response called SOS response [35]. In the presence of antibiotics pressure those bacteria will be favored that have acquired ARGs. It is not the matter of ARG only but the whole genome of that particular bacterium will be selected.

That is why genomic islands of the bacteria are being diversifying with the acquisition of resistance genes from environment and from other bacteria with the help of horizontal gene transfer [36]. For example a bacteria *Acinetobacter baumannii* has changed its genomic island and increase it by 66kb while it accepted 45 resistant genes and fix them into its genome. Hence this bacterium has transformed in 35 years from completely non-resistant to multidrug resistant. All of these genes are thought to be taken from the environment by the process of horizontal gene transfer [37]. Misuse of antibiotics and their release into natural environment have serious affects on the bacterial communities in natural environments. It leads to diminish in certain bacteria from the environment which are non-resistant and bacterial communities are losing their richness and only those bacteria are flourishing which are multi-drug resistant. Previous studies indicated that other natural environments like human airways, mammalian gut and animal husbandry facilities, where antibiotics are being used extensively are losing their bacterial richness in bacterial communities [38].

Under the selection pressure of antibiotics, bacteria are trying to acquire these resistance genes from other bacteria. So those bacteria having good contact rate, better ability of transferring genetic material, greater replication rates and having the greater ability to evolve are being favored naturally. In fact whole process of dissemination of antibiotics resistance is an ongoing example of Darwin theory of natural selection. As a result, the structure of whole micro biome is changing very quickly.

2.4 Presence of Antibiotic Resistance Genes (ARGs) in Natural Environment

Most of antibiotics are produce by soil-dwelling bacteria called the Actinomycetes (specially the Streptomyces). These bacteria produce antibiotics, including streptomycin, erythromycin chloramphenicol, tetracycline, and vancomycin. As these bacteria produce antibiotics, these bacteria must also be protected by the action

of these self-producing antibiotics. To protect themselves from the action of self-producing antibiotics these bacteria have developed antibiotic resistance genes in them. Today most of clinical important bacteria have these antibiotics resistance genes in them that were originally present in Actinomycetes [39]. However, it is noted that majority of streptomyces has show resistance against many drug classes. Atleast seven to eight antibiotics classes and leatest antibiotics practices are nullified by these streptomyces in now days [40]. It is matter of big concern that how single bacterium has become resisitant against so many antibiotics classes.

Bacteria have adopted themselves in a unique way that they can acquire antibiotic resistance genes not only from the environment through horizontal gene transfer but also they can acquire antibiotics resistance genes directly from their parents during the cell division in the process of vertical transfer [41]. However the horizontal gene transfer is of more importance because bacteria can acquire resistance genes from other bacteria and from environment which is a diverse source of antibiotics resistance genes [42]. These resistance genes are available for every bacterium that inhibits that environment, so there is no restriction in horizontal gene transfer.

By horizontal gene transfer bacteria can transmit not only their resistance genes but also whole plasmids that contain number of resistant genes. Resistance genes are accepted by the other bacteria because these resistance genes have little cost fitness that is why these resistance genes are easily be stabilized into new bacterium, hence spreading of antibiotics resistance is so fast[43]. These resistance genes provide protection against antibiotics but also these gene are involved in performing many internal functions of the cell. That is why these genes are always welcome by the bacteria [44]. In an opinion article by Martínez et al [45], described the ranking of resistance genes according to their potential risk of causing antibiotics resistance. These ranking are briefly described below.

RESCon 1: It includes resistance genes which are well known previously to confer resistance toan antibiotic and this gene is reported to present on mobile genetic elements and are presenthuman pathogen. Beside this, any novel resistance gene which is involve in inactivation of anAntibiotic which is also present on a mobile

genetic element hosted by a human pathogen i.e.New Delhi metallo--lactamase 1, NDM1.

RESCon 2: It includes novel resistance genes which is known to produce resistance against an antibiotic which is currently used in clinical practices and are also present on mobile geneticelements which is hosted by non-pathogenic bacteria. There is a greater chance of acquisition ofsuch resistance genes in human pathogens.

RESCon 3: It includes those resistance genes which confer resistance to a new antibiotic whichis in Phase I, II or III of its development. In this case antibiotic is used on limited scale.

RESCon 4: In this category those genes are placed which are well known to produce resist¬ance against an antibiotic in clinical, however the mechanism of resistance is not known. In this case resistance to this antibiotic is already caused by other resistance gene by a known mechanism.

RESCon 5: It comprises novel resistance gene that have a similar antibiotic substrate profile of already well known resistance gene. it don't include older genes but the novel genes only having resistance profile to already known resistance gene.

RESCon 6: If a gene that is predicted to produce resistance and it is present on mobile geneticelements. This gene has high sequence similarity with functionally characterized resistance genesand that gene is also present on mobile genetic element.

RESCon 7: It comprises those genes that are predicted to confer resistance and there is noassociation of this gene to any mobile genetic element. These genes have a certain degree ofsimilarity with functionally characterized resistance genes, however; further information aboutthis gene is not available. It don't includes novel resistance genes which is known to produce resistance against an antibiotic which is currently used in clinical practices and are also present on mobile geneticelements which is hosted by non-pathogenic bacteria.

2.5 Horizontal Gene Transfer and its Parameters

Horizontal gene transfer (HGT) is an important phenomenon for the dissemination of resistance genes. As a result of HGT resistance has expanded beyond the specific bacterial strains and resistant genes are available to a larger community of bacteria in a specific environment [46]. It is shown in a study that resistant gene and host bacteria must share at least temporary the same habitat [47]. It is also noted that bacteria which are phylogenetically closely related, can share the resistant genes more easily by the process of HGT [48]. Following parameters should be considered for the successful horizontal gene transfer and dissemination of antibiotics resistance. Contact rate is the first main parameter of HGT. In this

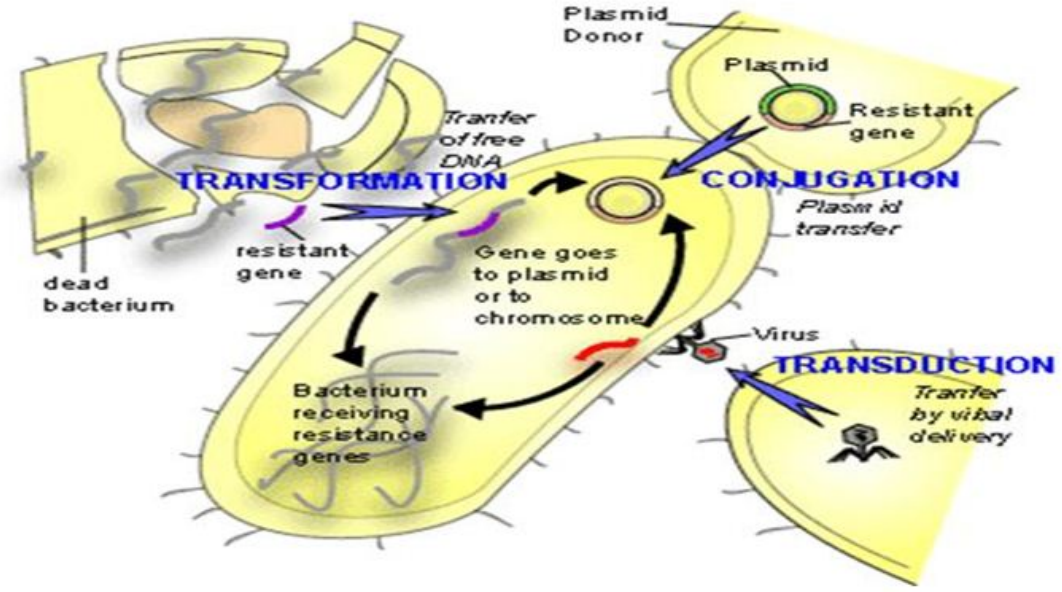


FIGURE 2.2: Mechanisms of horizontal gene transfer in bacteria

case there are two types of cells, one that possess resistance gene and other that need to acquire this resistance gene. For the better contact rate population size of both types of cells must be large. Second parameter for HGT is transfer rate of genetic material. Interaction or contact rate which is previously described, between the donor and recipient cell is not sufficient. Contact of donor and recipient cells would be useless if the transfer of genetic material is not take place. The

transferability of the resistant gene is influenced by the rate of transfer of plasmids, rate of transfer of integrative-conjugative elements, or chromosomal fragments (including islands) into recipient bacterial cells; 2) transfer of resistance genes from one plasmid into other plasmid or from plasmid into the chromosome; 3) transfer of chromosomal genes into plasmids; 4) transfer of transposons into plasmids or into the chromosome. The third parameter is integration rates. It involves the stabilization ARGs into recipient cell. The integration involves the expression of resistant gene in to recipient cell [49].

This integration requires that the regulatory and metabolic machinery of the recipient cell must be compatible with the incoming resistance gene [50]. The fourth main parameter is replication rates. As the bacterial cell divides more quickly its population size will also increase rapidly. It is also necessary parameter that is involved in the spread of newly taken resistance gene, so that gene amplification takes place within the same population of bacteria [51]. The fifth main parameter is related to diversification rates. It refers to evolvability of the acquired resistance genes. In this regard we must consider the mutation frequencies or, better, mutation rate per gene per cell and generation, inducible mutation rates, rates of clonal diversification within clonal complexes, recombination rates, colonel diversification into defined ecotypes, and migration [52]. Sixth parameter is selection rates. It depends upon type of ARG and selection forces i.e; presence of antibiotics and heavy metals.

2.6 ARGs in Air Particulate Matter

In recent years the attention is diverted to investigate the presence of ARGs om air particulate matter. By carefully reanalyzing the reported sequence data, Pal et al. (2016) indicated that Beijing smog posses those bacteria that have a higher richness of 64.4 ARG sub-types than any other environments, for example, pharmaceutically polluted environments have 38.9 , 19.4 for wastewater, 11.8 for animals, 1.0–16.6 for humans and 1.6–3.3 for other terrestrial sources [55]. There is

not only seasonal variation in the presence of ARGs on the particulate matter but concentration of ARGs also varies at different time within the same day at same location. In a previous research [18], global survey of ARGS was conducted by a team scientist. In this research a total of 39 ARGs were analyzed across 19 cities of the world. They have detected 30 ARGs in air particulate matter. Among these 30 ARGs, 18 ARGs were present in the air particulate matter of Beijing and it was the highest number of detected ARGs in comparison to all other cities.

The top two most abundant ARGs in global particulate matter were bla TEM and qepA, which provides resistance to -lactams and quinolones respectively [56]. They analyze the antibiotics consumption data of 5 major antibiotics including -lactams, quinolones, macrolides, tetracycline, and amino glycosides,[57] and found a linear positive correlation between the global antibiotics use patterns and the abundance of air born ARGs correlation (Pearsons' r = 0.989, p-value = 0.001). Its means use of antibiotics is responsible for the relative abundance of ARGs in air particulate matter. In another study the quantity of blaTEM and qepA was measured in air particulate matter of china, from the year 2004 to 2014. It was found that the relative abundances of blaTEM and qepA had increased by 178 percent (p-value = 0.008, Paired t test) and 26 percent (p-value = 0.483, Paired t test) respectively. Increase in ARGs with the use of respective antibiotics is well supported in this case by anchor study. Sun and Wang in (2012) indicated that the daily defined doses of the cephalosporins (-lactam) and levofloxacin (quinolone) have increased by 427 percent and 109 percent in the central Hospital of Xi'an Huashan, from the year 2008 to 2010 [58].

In a recent research it was shown that at in the month of February when the particulate matter was low, 29 ARGs were detected. Among them top 8 ARGs subtypes were blaTEM, tetW, sul3, tetQ, tetM, ermB, tet32, and tetO. Out of these 8 ARGs five ARGs were tetracycline resistance genes [53]. Beside this it was also noted in their study that the quantity of ARGs also varies at different times within the same day. Sulphonamide resistance gene sul3 increase sharply at 20.00 and its concentration increased up 40.96 percent of the total ARGs that was

comparable to blaTEM concentration. However, after four hours the concentration of sul3 decreased substantially back to normal.

On the other hand Tetracycline resistance gene tetW, decreased suddenly during this time period. It is very surprising to note here, the concentration of one ARG was increasing and the concentration of other ARG was decreasing at the same time. On March 11, 2018 at 8:00 early morning time, when pm concentrations was high the most prevalent ARG was NDM-1 that account for up to 30 percent of the total ARG abundance and its concentration was 54g/m3. However at 20:00 concentration of this gene decreased to about 20percent. On next days, March 13, 2018, the NDM-1 gene concentration increase again early in the mornings and noon up to 251g/m3. These findings suggest that there is not only seasonal variation in the concentration of ARGs but also variations do exist within the same day at different times.

In this study they observed that intI1 gene and tnpA gene also varied with time at different levels of PM2.5 (p-value=0.006 and p-value=0.05,). For Feb 6 to 7 at low PM episode, they have detected a substantial increase in the relative abundance of intl1 gene at 24:00. On the other hand for March 10–13, when pm concentration was high, the relative abundance of tnpA gene was much higher than the intl1 gene and the highest concentration was observed at 04:00 on March 11. It is seen that the concentration of the intl1 gene and tnpA gene not only were different for high haze and low haze days but also differ greatly across the time within same day. These results showed that different bacterial activities were taking place for different PM levels [53]. In another study it is shown that the intI1 gene was present in all PM samples, while tnpA was only present in the samples of the spring of 2009 [54].

2.7 Bacterial Population in Air

Bacteria are present in the atmosphere with PM in the form of spores, vegetative or dividing cells [59, 60]. Beside this smaller bacteria can be present in the air

for many days and they can be transported easily over long distances. It was noticed that bacterial viability also change with the levels of air PM and can be increased up to 60 percent at some point during a heavy haze episode in Beijing. Presence of large number of bacteria and their metabolic activities can alter the chemistry of aerosols [61]. During the past decade, bacterial community richness and bacterial diversity has increased in the winters. In summers the bacterial populations has changed during last decade but in summers despite the batter conditions for growth, loss of bacterial diversity is seen in PM borne bacterial communities.

Mao Y., et al conducted a study in Beijing and found that in polluted weather concentration of bacterial cells was significantly higher as compared to the concentration of bacterial cells in weather with less pollution[62]. The bacterial composition in the air is correlated with the pollution levels of air, and gram negative bacteria GNB were more abundant. Beside this they have found that, the relative abundances of many bacterial genera alters as the level of smog changes, for example, during heavy-smog days bacterial genera like *Methylobacillus, Desulfurispora, Tumebacillus* has increased in their concentration. One fourth of the bacterial genera were found to be multi-antibiotic resistant, which may serve as a potential pathway for antibiotic resistance transfer between bacteria. It was also found that More than 70 percent bacterial isolates were Penicillin resistant. The presence of multi-drug resistant bacteria in enormous amounts indicates a potential risk of transfer of resistance to human pathogens [62].

In another study that is conducted in Beijing and Shijiazhuang, bacterial populations on particulate matter was studied [63]. In this study it was found that Bacillus spp are more abundantly found among the culture able isolates of bacteria. Other culture able bacterial isolates include *Microbacterium esteraromaticum, Planococcus dechangensis,Kocuria rosea*. For Shijiazhuang isolates it was seen that Bacillus *halotolerans* (SJZ) hosted the large number of ARGs subtypes, greater than all other bacterial species. *B. halotolerans* also hosted the NDM-1 and this gene is also contributed to the particulate matter predominantly by this bacterial specie in Shijiazhuang. On the other hand, they have found that sul3 gene was

widespread among the culture able bacterial isolates that were present in Shijiazhuang, blaTEM and tet32 genes seconded the sul3 gene in their spread among the bacterial isolates. In this study it was found that most of the screened ARGs are present in bacillus while SJZ also carried many ARG subtypes. tnpA gene and intI1 gene was primarily contributed by *B. halotolerans* and Bacillus species *Bacillus marisflavi*(SJZ) respectively. In another work, it was shown that during the high-haze episode, airborne bacterial communities varied greatly with time, and *Massilia* and *Acinetobacter* alternated with one another as a major dominant bacterial species with an abundance of up to 90

2.8 Inter Conection Between Antibiotics Resistant Bacteria and Antibiotics Resistance Genes

In recent years we are facing new multiple challenges that are also interconnected with one another. Increasing levels of Smog and dissemination of antibiotics resistance are current challenges which are interlinked. Air particulate matter provides a physical surface to which Antibiotics resistance genes and resistant bacteria can adhere more stably. During the smog concentration of air particulate matter increase so many times, that is why more antibiotics resistance genes and bacteria are present in smog and heavy haze air. Bacteria can acquire antibiotics resistance genes form particulate matter and can transfer to other bacteria through horizontal gene transfer. By considering the parameters of HGT and risk factors of ARGs we can distinguish more potent ARGs and bacteria which are involve in dissemination antibiotic resistance. Due to miss use, large amount of antibiotics are released into natural environments. These antibiotics create a selection pressure on bacterial populations and those bacteria survive which posses ARGs.

In last decade the use of cephalosporins (-lactam) and levofloxacin (quinolone) has increased. In different studies it was shown that blaTEM and qepA genes are most abundant ARGs in air particulate matter. BlaTEM and qepA genes provides resistance to -lactam and quinolone. Hence it is obvious that in the presence of a

cephalosporins (-lactam) and levofloxacin (quinolone) antibiotics, bacteria rushes for acquiring those specific ARGs that confer resistance against these antibiotics. In this situation those bacteria having good contact rate and better conjugative machinery will be favored. We have seen that bacillus and gram negative bacteria dominate the bacterial populations during smog.

Due to better growth rates there is a higher chance that these bacteria are involve in the spread of blaTEM and qepA genes. Anthropogenic activities from the ground and bacterial activities changes continuously during a day. That is why the concentration different ARGs also vary. Inhalation of ARGs with air particulate matter during smog can create serious threats of dissemination of antibiotics resistance in human microbiota, especially when a person is going through antibiotic therapy. Other consequences of inhalation of air particulate matter are still need to be investigated.

Chapter 3

Material and Methods

3.1 Samples Collection

Particulate matter samples were collected from two different sites, H9 and Kahuta industrial Areas near the highly populated areas in Islamabad. PM2.5 samples were collected at a flow rate of 1000 L/min by using digital portable high-volume air samplers, fitted with fiber glass filters having the size of 19.0 cm$\times$23.8. Samples were collected 2-3 meters above the ground surface. All the apparatus especially Filter cartridges was washed carefully with 70 percent ethanol to avoid any contamination. Sampling took place 24 hours, for the smog and none smog days. The sampling was done in month of December 2019 for smog sample and for non-smog sample the sampling was performed in May 2020. After sampling each filter sample was sealed in sterile plastic bags and stored at 20 °C in the university laboratory until we processed these samples further. We cut the all filter papers of both samples in to small pieces and mix the filters of both sides separately for smog and non-smog samples. After mixing these we have taken 1/4 part of these filter papers and make inoculum for bacterial growth. While 3/4 part of the smog sample was preserved at 20 °C for DNA extraction.

3.2 Culturing of Bacteria

Process for bacterial isolation was same for both smog and non-smog samples. Filter papers were crushed in to very small pieces; we take 1 gram filters and dissolved them in 10ml double- de-ionized and autoclaved water. This makes our stock inoculums for bacterial isolation. Next we have performed serial dilutions of our sample and selected the serial dilutions No -3 to -7 for inoculation. We have used two types of general growth media i.e; LB agar, TSA agar and MacConkey agar. We inoculated the bacteria on agar plates by spreading method and incubate the culture plate at three different temperatures i.e; 25 °C, 37°C and 42°C. After 24 hours. We started screening of our plates and bacterial colonies which were showing different characteristics were picked and purified on separate plates. After purifying we closely monitor the characteristics of bacterial colonies.

3.3 Antibiotic Susceptibility Test

We use the Kirby-Bauer disk diffusion method to detect the antibiotics susceptibility. The following antibiotic discs were used: tetracycline 25 g, erythromycin 15g, vyncomycin (VAN) 10g, ampicillin (A) 30g, naladic acid 30g(N.A) and imipinum(10g). These antibiotics were selected because they are belonging to different classes of antibiotics and either used extensively in antibiotics therapies. After making the pure colonies of bacterial isolates we pick a single colony from culture plate and dissolve it in saline solution and mix this colony thoroughly on vortex mixer.

After mixing these colonies we take a sterile cotton swab and dip into our sample and gently swipe this cotton swab on the surface of culture plate having Mueller-Hinton agar. We incubated the plates on 37°C. after 24 hours we start screening the plates and trying to identified resistant bacteria on the bases of inhibitory zone. We have identified 5 most resistant strain from both smog and non-smog

sample, identified them by whole genome sequence. For genome sequences we send our samples Macrogen clinical Laboratory Korea.

3.4 DNA Extraction

In our study we insure that external DNA that is bounded to particulate matter is extracted and it do not contain the DNA of lysed cells. So we enable to detect ARGS which are purely present on particulate matter. We started DNA extraction by socking all the glassware used in the experiment in 1 M NaOH, and 10 percent HCl The glassware utilized for nucleic acid analysis was carefully cleaned by soaking it in 1 M NaOH, 10percent HCl. We wash the all glassware with doubled-ionized water to remove any organic contamination. After this all glassware is autoclave at high temperature of 250°C to stop any kind of nuclease contamination. Now we carefully remove dust particles from the filters and added 1 gram P.M into 9 ml of 0.1 M sodium phosphate buffer at pH 8.0 in falcon tube.

Now we added 0.5 g of acid-washed polyvinylpolypyrrolidone PVP to the mixture. We set the speed of horizontal shaker at 150 horizontal shakes per minute and place our sample on the shake rfor 1 minute. This mixing step was repeated three time for 1 minute break at each time. During the break of 1 minute the sample was placed in chiller. Now we added very small amount of Sodium dodecyl sulfate (SDS) at final concentration, 0.1percent was added, and the sample was shaken again for 10 s.

Now we have chilled the samples on ice and performed low speed centrifugation at 500 $\times$ g for 10 min only. The temperature was maintained at at 4°C. We have collected the supernatant and transfer it into a sterile tube. Pellet was washed two more time and the supernatants were collected. In this step we don't have used SDS because it can disrupt the cell membranes of bacterial cells. All three supernatant were combined and subjected to high speed centrifugation. The speed of centrifugation at this time was selected at 10,000 $\times$ g for 20 minutes at at 4°C.

We added now adding 1 volume of a C-etyltrimethylammonium bromide CTAB solution to precipitate the DNA. We make a buffer solution of 50 mM Tris to10 mM EDTA, pH 8.0 and use this buffer solution to make 1 CTAB. Now we incubated the sample at 65°C for 30 min and then centrifuged again at 5,000 × g for 10 min at 4°C. as the DNA is precipitated we discarded the supernatant now and pellet was dissolved in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, 1 M NaCl; pH 8.0). Then we 0.6 volume of cold isopropanol to the sample, and the sample was incubated for 1 hour on ice. We perform high speed centrifugation at 10,000 × g for 15 min at 4°C.

We resuspended the pellet in 10 mM Tris-HCl-0.1 mM EDTA at final pH 8.0and an equal volume of phenol-chloroform-isoamyl alcohol in ratio of 25:24:1 by volume was added to each preparation, and the we perform high speed centrifugation at 10,000 × g for 5 min. We separate the supernatant and add equal volume of chloroform-isoamyl alcohol in a ratio of 24:1 by volume and centrifuged again at the same speed. We separate the supernatant. Then we add cold 70 percent cold ethanol to this supernatant and added sodium chloride with final concentration, 0.2 M. now we have incubated the sample at 20°C for the time of 1 h hour, and perfume the centrifuge again at the speed of 10,000 × g for 15 minutes. We discard the supernatant and pellet was washed with ethanol and dried under vacuum. Finally we have the pellet in double de-ionized autoclaved water.

3.5 Identification of ARGS in Extracellular DNA

We have chosen 5 antibiotics resistant genes and one intigron gene because of their clinical importance, and on the bases of antibiotics prescription patterns. Our aim is to detect these ARGS in Edna. We have constructed their primers and done PCR separately with extra chromosomal DNA samples. As the target gene is amplified after PCR, it will confirm it presence in the extra chromosomal DNA. Selected genes and their primers are given in tab2. To check the product of PCR, we run the products on agrose gel by the following protocol. First of all we added

basic blue dye to our PCR products so that they can be visualized. We made the gel by adding the 1 gram of agrose in 100ml of TAE buffer. Basic principle of this protocol is that the DNA has negative charge.

When electrodes are applied across the gel and electricity is given due to negative charge on the anode will push the fragments of DNA towards the positive cathode. If the size of the DNA fragment is smaller it will run faster than the others. Hence these fragments become separated. We loaded the PCR product in comb one by one separately for each PCR product. A DNA ladder of know base pairs is also loaded on the gel as a reference. As the electricity is provided blue dye started movement from the comb chamber to the other side. After 1 hour of eclectic supply we switch off the electric current. I turned off the power supply and the gel is removed and placed into an ethidium bromide solution. Between the DNA the ethidium solution intercalates and visible in uv lights sometime it is directly added to the Agrose gel solution. After that I exposed the stained gel to uv light and then image is taken. DNA bands are visualized. The loaded DNA ladder is also visualized and length of DNA bands was estimated.

Table 3.1: antibiotics resistance genes and their primer

Genes	**Primers**
blaTEM(forward)	ACGCTCACCGGCTCCAGATTTAT
blaTEM(Reverse)	TCCTCCGATCGTTGTCAGAAGT
tet M(Forward)	CATCATAGACACGCCAGGACATAT
tet M(Reverse)	CGCCATCTTTTGCAGAAATCA
sul3(forward)	GAGCAAGATTTTTGGAATCG
sul3(Reverse)	CATCTGCAGCTAACCTAGGGCTTTGGA
vanA(Forward)	AAAAGGCTCTGAAAACGCAGTTAT
vanA(Reverse)	CGGCCGTTATCTTGTAAAAACAT
qnrA(Forward)	ATTCTCACGCCAGGATTTG
qnaR(Reverse)r	GATCGGCAAGGTTAGGTCA
Intl 1(Forward)	CGAACGAGTGGCGGAGGGTG
Intl 1(Reverse)	TACCCGAGAGCTTGGCACCCA

3.6 Procedure for Identification of Most Resistant Strains

Genomic DNA was extracted from fresh cultures using the phenol–chloroform method. We construct universal primer (27F) 5 AGA GTT TGA TCM TGG CTC AG 3. We did PCR separately for each strain. We amplify their target sequence and compare the target sequence in NCBI database sequences with the help of bioinformatics tools. We identified the most resistant bacteria at specie level.

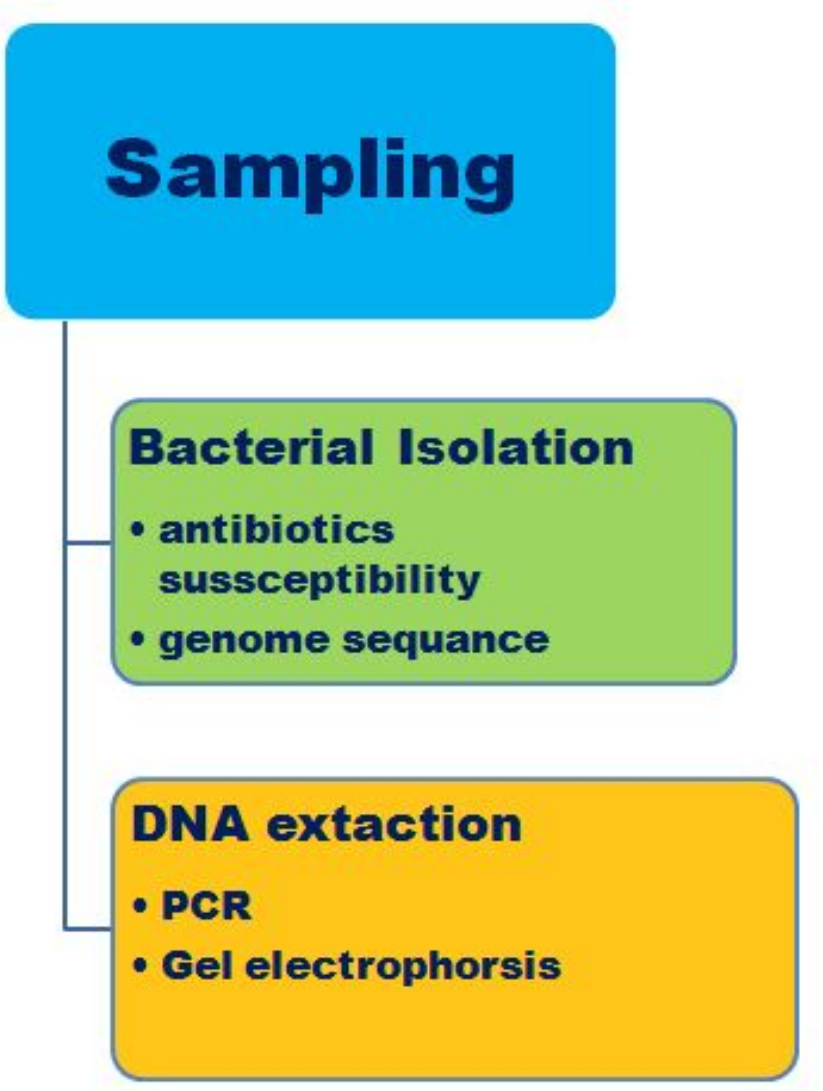


FIGURE 3.1: methodology of research

Chapter 4

Result and Analysis

4.1 Bacterial Isolation for Smog Sample

We closely observed various characteristics of bacterial colonies such as, color, shape, and elevation from surface, growth rate and optimum temperature for growth. After careful observations we have identified 27 different colonies of these bacteria. Most of the stains were off-white color, while some strains have yellow color and others have brown color. Some strains were fast growing and they have produce colonies in 24 hours. These strains are termed as fast growing. Other strains produce colonies after 48-72 hours and their growth are termed as medium. Still other has slow growth and they show growth after 72 hours. Beside this, the optimum temperature for the growth for different bacterial strains was also different. Majority of bacteria 55.56 percent were growing best at 25°C, while others 33.33 percent are growing on 37°C and some are growing at 42°C. Most of the bacterial colonies were round while some oval and shaped colonies were also present. Size of colonies also have variations, some are larger some are medium sized and some are smaller or pointed. Some colonies were close to surface some are slightly raised and some are raised well above the surface. Characteristics of different bacterial strains that were isolated from smog sample are given in the table 4.1 below.

Table 4.1: Bacterial isolates from smog sample

Name	Color	Shape	Elevation	Growth	Temperature
S1	White opaque	Round large	Slightly raised	Fast	25
S2	White transparent	Round small	Slightly raised	Slow	37
S3	White opaque	Round small	Closs to surface	Medium	25
S4	Yellow bright	Round small	Slightly raised	Fast	37
S5	White opaque	Round large	Close to surface	Fast	25
S6	Brown bright	Round small	Slightly raised	Fast	37
S7	Yellow bright	Round large	Raised	Fast	37
S8	Off White opaque	Star shape	Closs to surface	Fast	42
S9	Off White opaque	Round small	Slightly raised	Fast	25
S10	Yellow bright	Round pointed small	Closs to surface	Slow	42
S11	Light yellow	Round small	Slightly raised	Medium	25
S12	White bright	Round large	Close to surface	Fast	37
S13	Light yellow	Round small	Close to surface	fast	25
S14	Off white opaque	Round small pointed	Slightly raised	Medium	25
S15	White transparent	Round Large	Raised	Fast	25
S16	Yellow opaque	Round medium	Closs to surface	Medium	25

S17	Off white opaque	Star shape small	Raised	Medium	25
S18	White opaque	Round small	Raised	Slow	37
S19	Slightly yellow	Round medium	Closs to surface	Medium	25
S20	Off white bright	Star shape	Slightly raised	Fast	25
S21	Yellow bright	Round medium	Slightly raised	Medium	25
S21	White opaque	Round small	Closs to surface	Medium	42
S22	Slightly yellow	Round large	Closs to surface	Medium	37
S23	White opaque	Wavy large	Closs to surface	Fast	37
S24	White transparent	Round small	Slightly raised	Medium	25
S25	Slightly yellow	Round small	Raised	Medium	37
S26	Off white opaque	Round medium	Closs to surface	Fast	25
S27	Dark yellow bright	Round medium	Raised	slow	42

It was earlier mention that bacteria are present on the particulate matter in various forms like spores, vegetative cells or as dividing cells. Size of the bacteria also varies, some are larger bacteria and some are smaller. However; smaller bacteria that are present on particulate matter can remain suspended on particulate matter in several days and they can travel across long distances from their sit of aerosol to other places. It is also noticed that viability of bacteria on particulate matter also varies with the concentration of particulate matter in the air. More the particulate matter in the air more is the chance of bacteria to survive. In some studies it is found that the bacterial viability on particulate matter has increased 60 percent [61]. However in our studies there is no strong evidence regarding the high prevalence or viability of bacteria on air particulate matter when particulate matter concentration is high in smog samples. There are two possible reasons: (1) our studies are based on cultivable bacteria, and these bacteria are only a fraction of all bacteria that are present on particulate matter, so a meta-genomic analysis may give a clear picture in this regard, (2) environmental conditions and factors are not so good for the bacteria during smog season. During the smog season although the particulate matter is more and it provides more site for bacteria to adhere themselves but other environmental factors like temperature and humidity are not so good. There is low humidity and low temperature during the smog season, so it can be a possible reason that we don't have find any strong difference in viability of bacterial isolates. However; we have found that most of the bacterial isolates in smog sample are growing at 25°C, while in none smog sample we have found that there are relatively less bacterial isolates were growing on 25°C. This indicates that the most of bacteria that grows on higher temperature are missing in smog sample. This finding also indicates that during the smog season when the particulate matter is higher only those bacteria have high prevalence which can tolerate high stress of temperature and humidity. It means that in smog those bacteria are present on particulate matter those are stronger than the others and hence when they cause infections to human beings; it is difficult to treat such bacteria. It is seen that during the smog season people have serious respiratory infection which are difficult to treat; this is according to our studies. pictures of

pure isolated colonies are attached bellow.

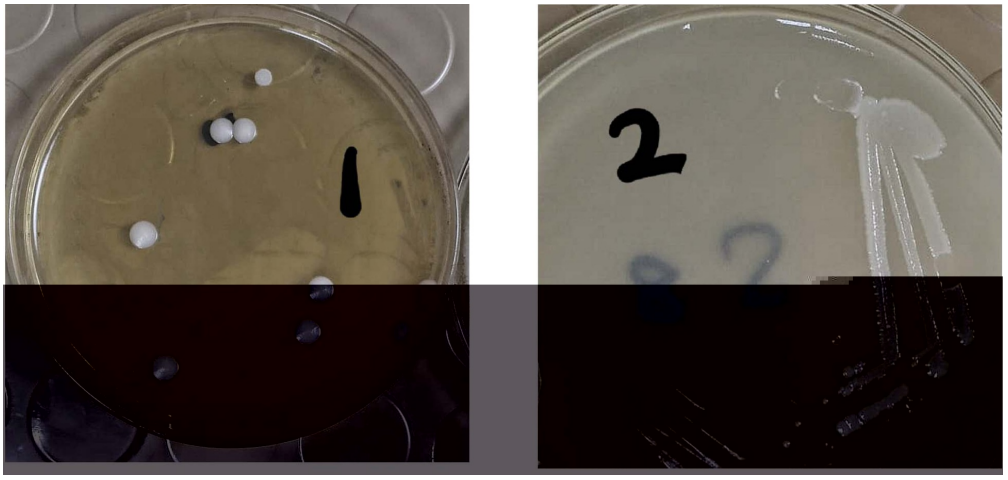


FIGURE 4.1: Bacterial isolates from smog sample S1 and S2

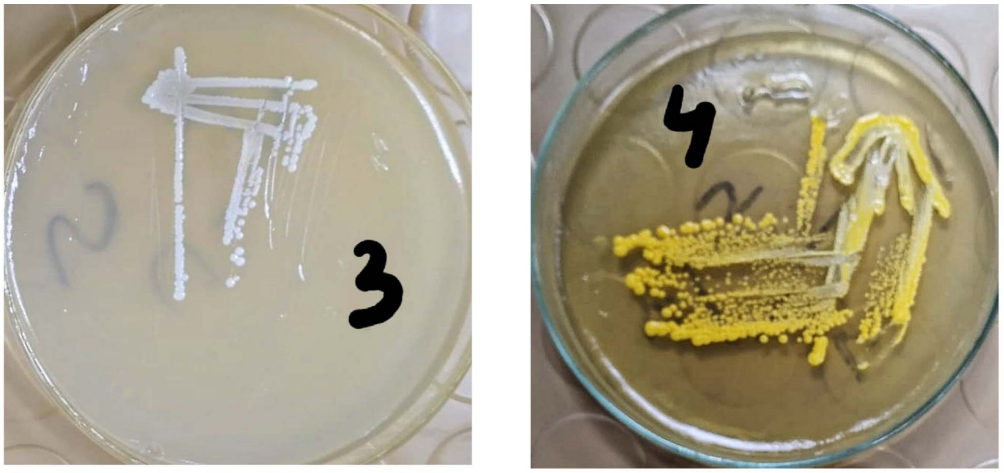


FIGURE 4.2: Bacterial isolates from smog sample S3 and S4

FIGURE 4.3: Bacterial isolates from smog sample S5 and S6

FIGURE 4.4: Bacterial isolates from smog sample S7 and S8

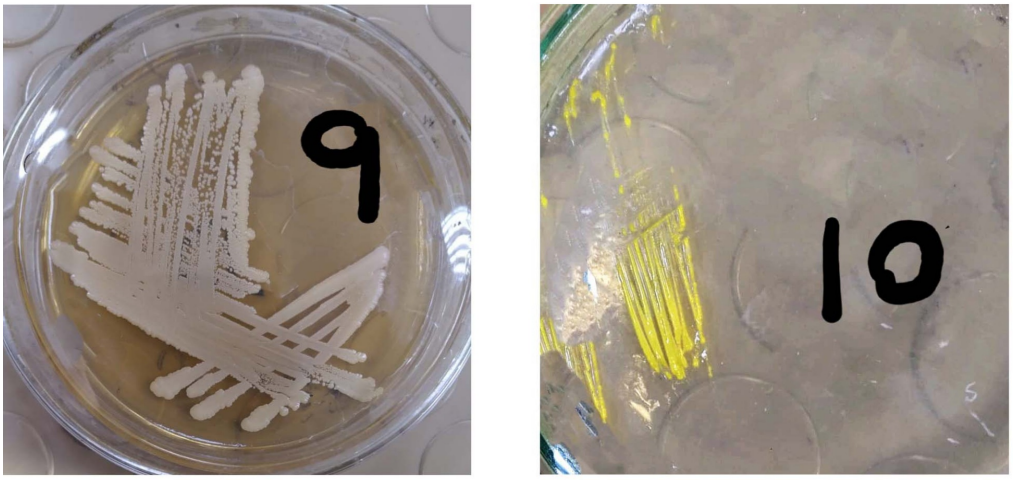


FIGURE 4.5: Bacterial isolates from smog sample S9 and S10

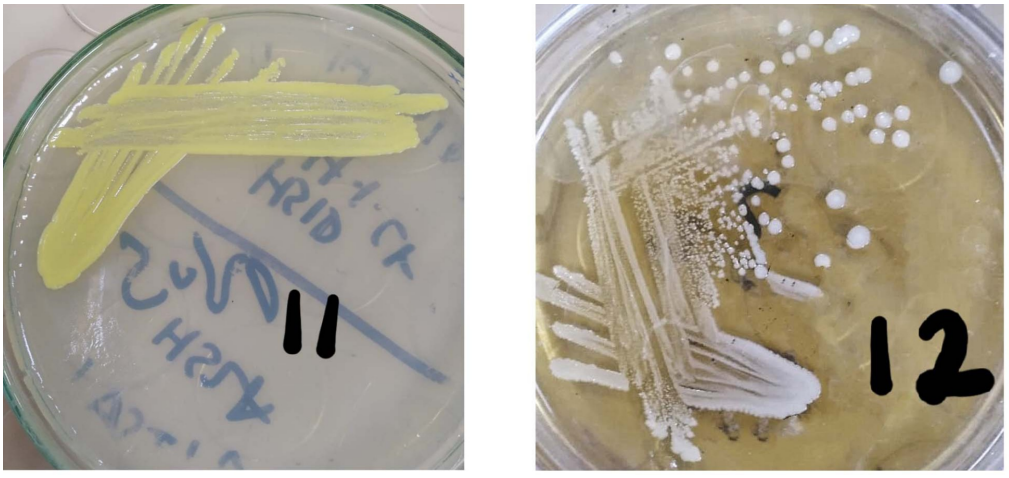


FIGURE 4.6: Bacterial isolates from smog sample S11 and S12

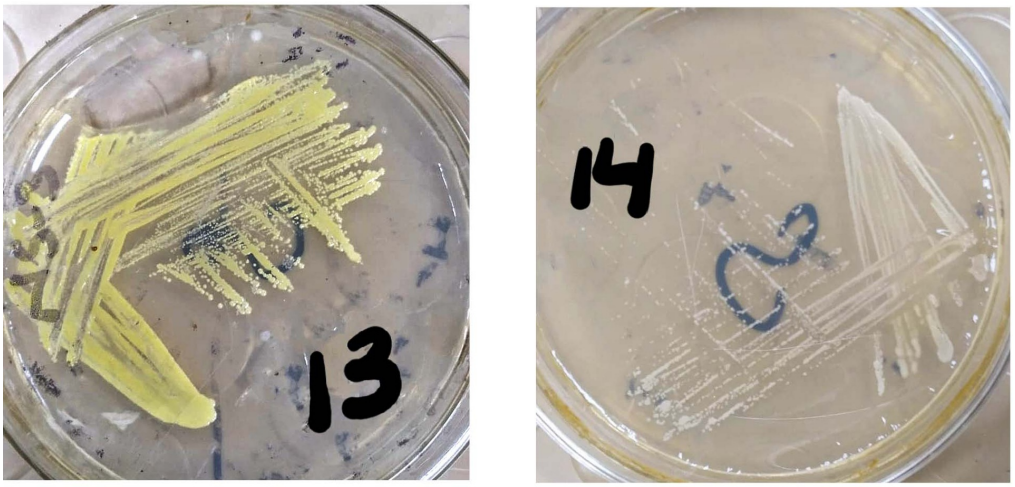


FIGURE 4.7: Bacterial isolates from smog sample S13 and S14

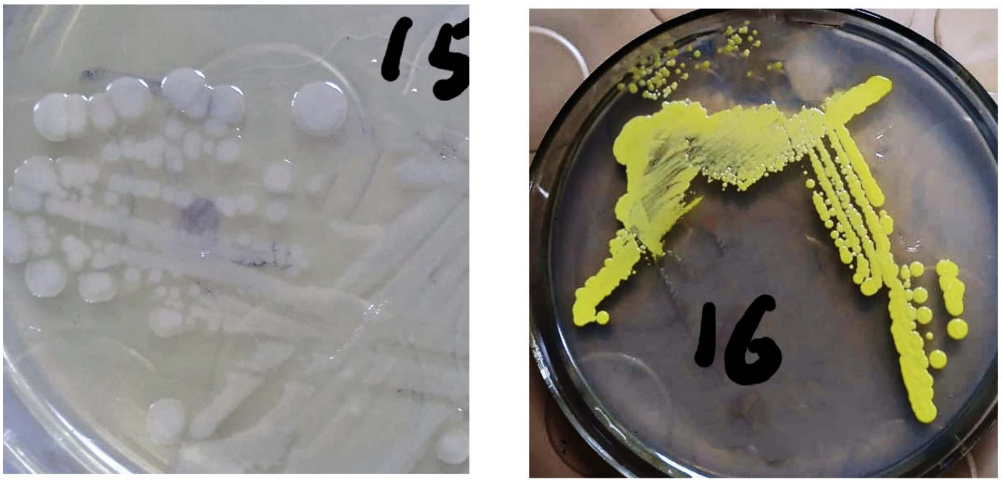


FIGURE 4.8: Bacterial isolates from smog sample S15 and S16

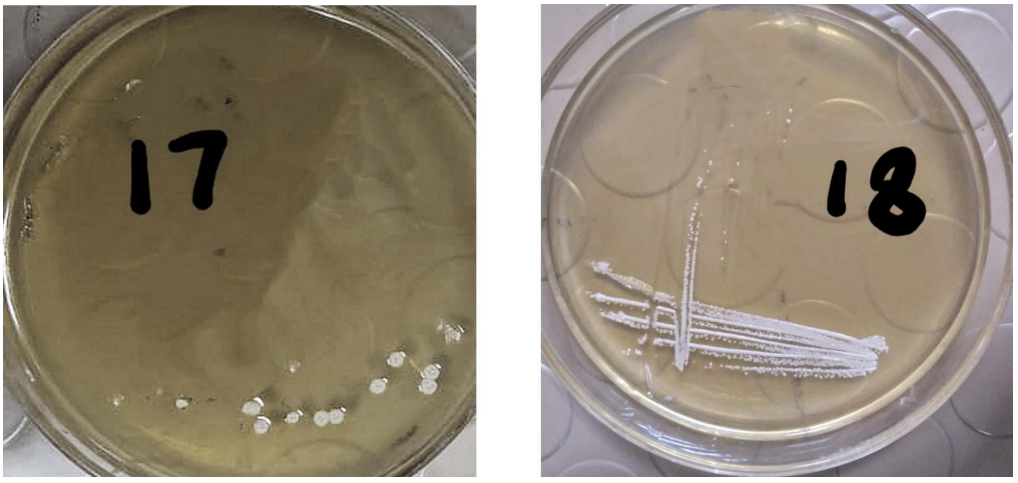


FIGURE 4.9: Bacterial isolates from smog sample S17 and S18

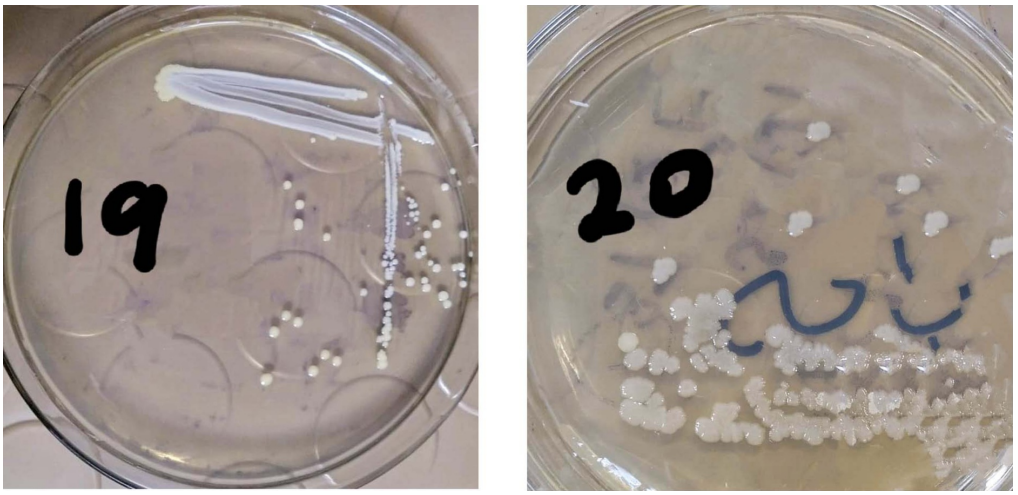


FIGURE 4.10: Bacterial isolates from smog sample S19 and S20

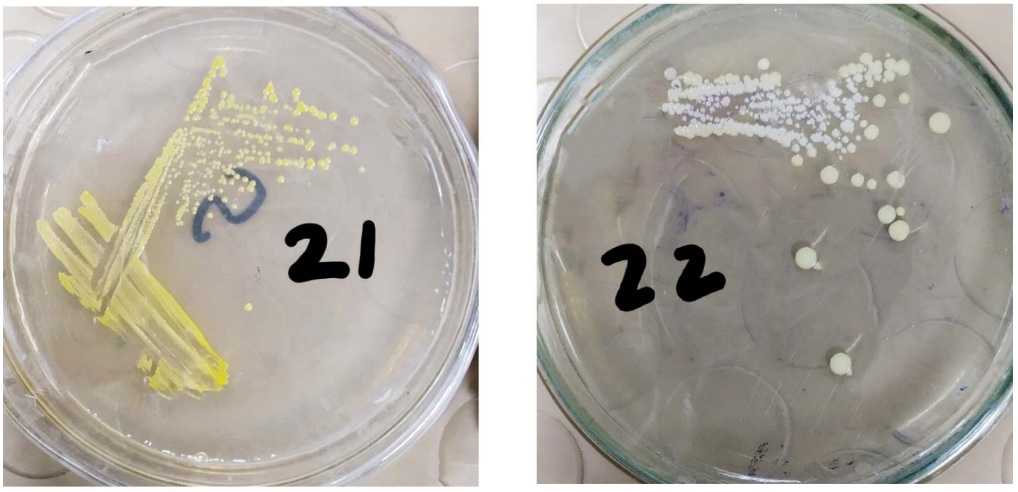


FIGURE 4.11: Bacterial isolates from smog sample S21 and S22

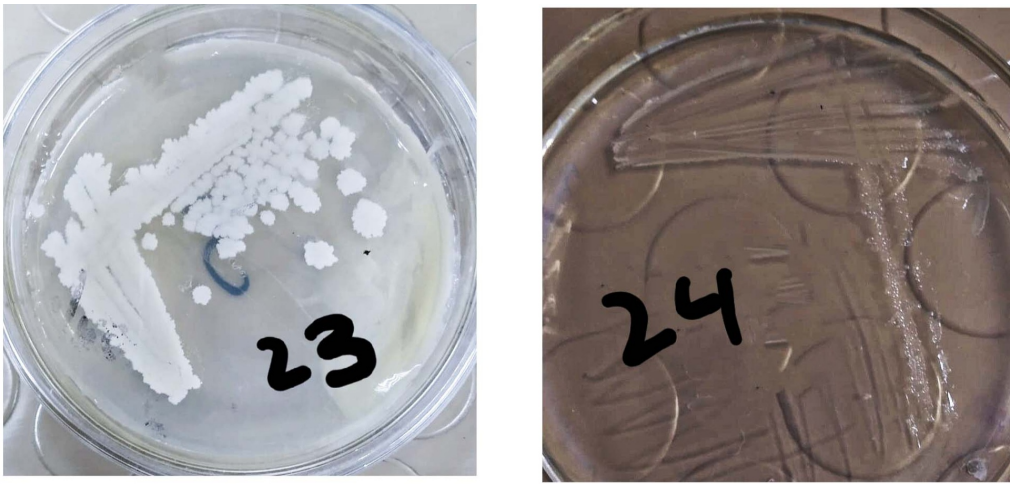


FIGURE 4.12: Bacterial isolates from smog sample S23 and S24

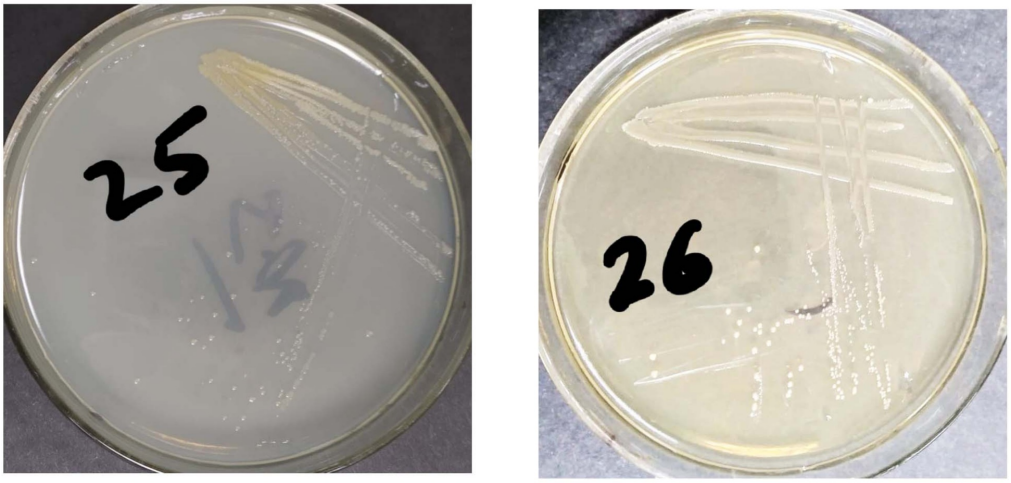


FIGURE 4.13: Bacterial isolates from smog sample S25 and S26

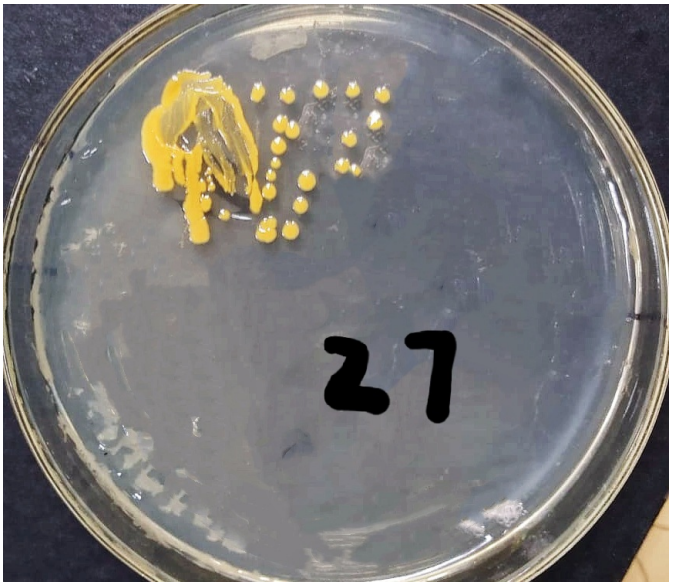


FIGURE 4.14: Bacterial isolates from smog sample S27

4.2 Bacterial Isolation for Non-Smog Sample

We have isolated 25 cultureable bacterial strains in non-smog sample. Most of the stains were off-white color, while some strains have yellow color and others have brown color. Some strains were fast growing and they have produce colonies in 24 hours. These strains are termed as fast growing. Other strains produce colonies after 48 -72 hours and their growth are termed as medium. Still other has slow growth and they show growth after 72 hours. Beside this, the optimum temperature for the growth for different bacterial strains was also different. Some

bacteria 36 percent were growing best at 25 C, while others 48 percent are growing on 37 C and some are growing at 42C.

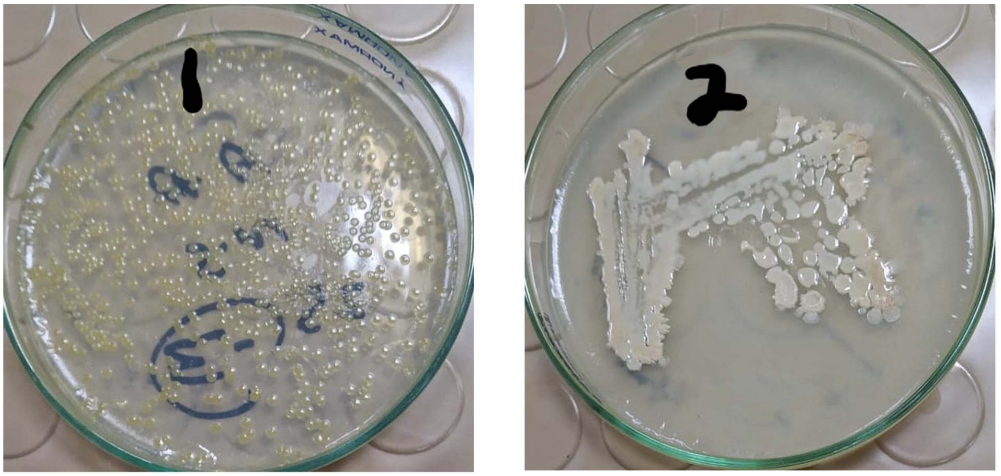


FIGURE 4.15: Bacterial isolates from smog sample N.S1 and N.S2

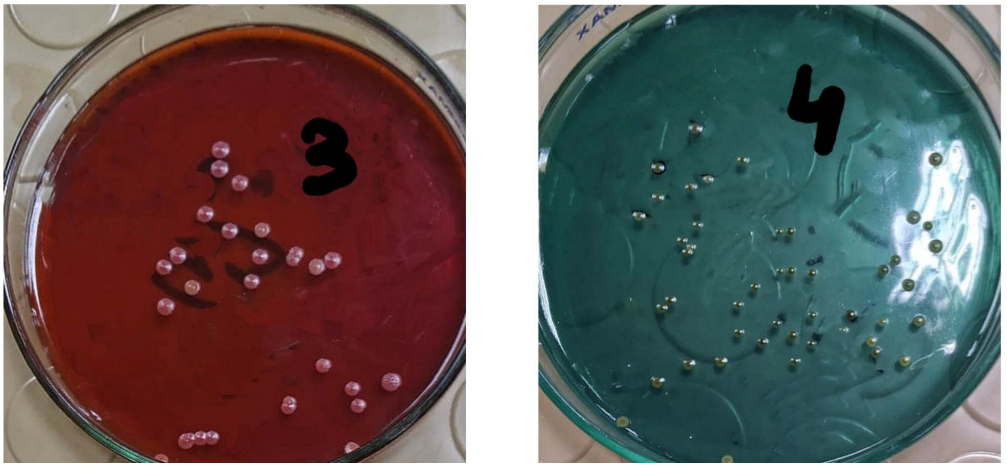


FIGURE 4.16: Bacterial isolates from smog sample N.S3 and N.S4

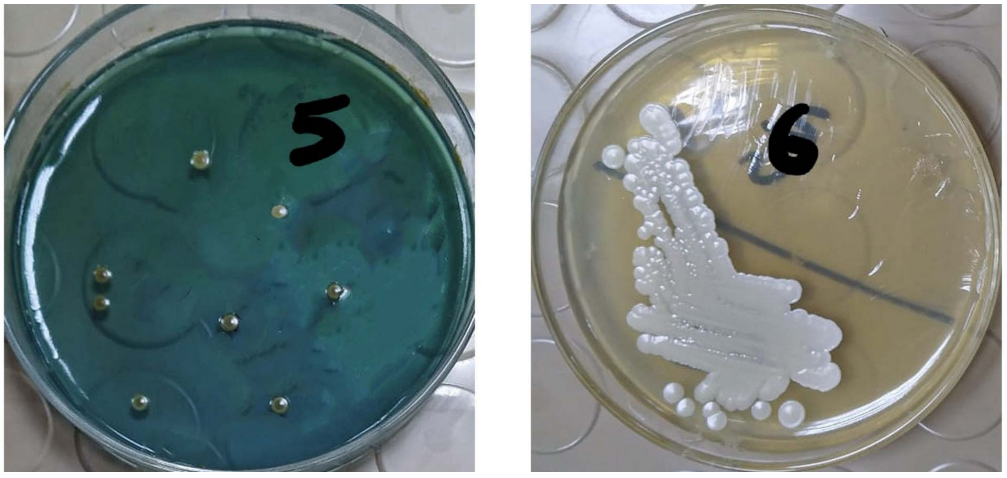


FIGURE 4.17: Bacterial isolates from smog sample N.S5 and N.S6

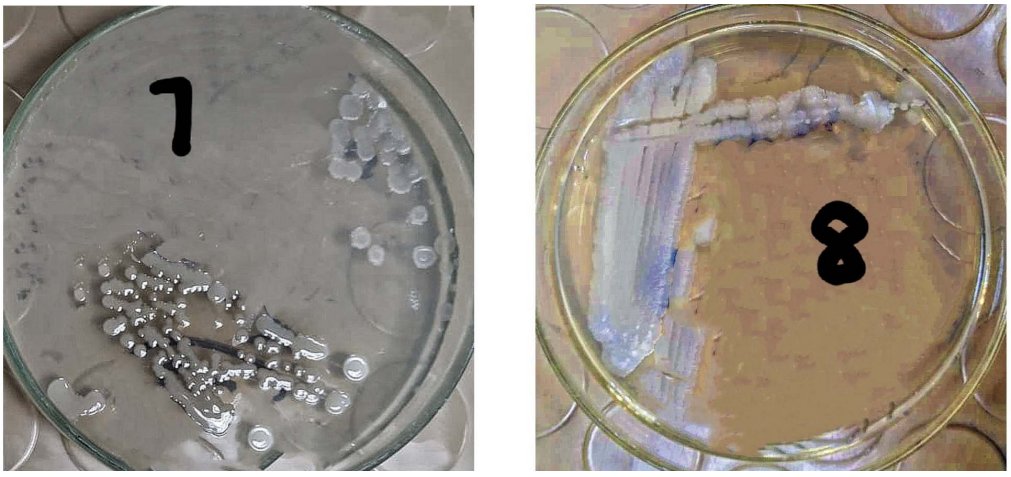


FIGURE 4.18: Bacterial isolates from smog sample N.S7 and N.S8

FIGURE 4.19: Bacterial isolates from smog sample N.S9 and N.S10

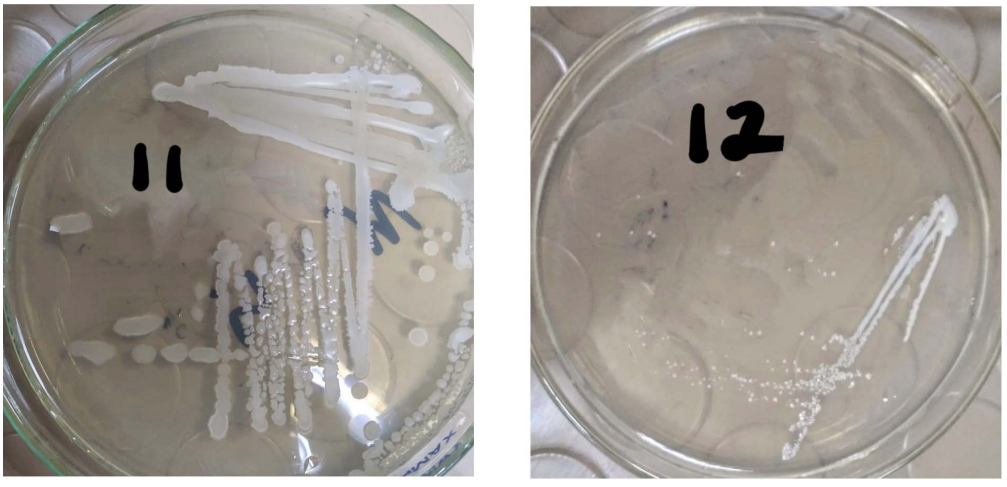


FIGURE 4.20: Bacterial isolates from smog sample N.S11 and N.S12

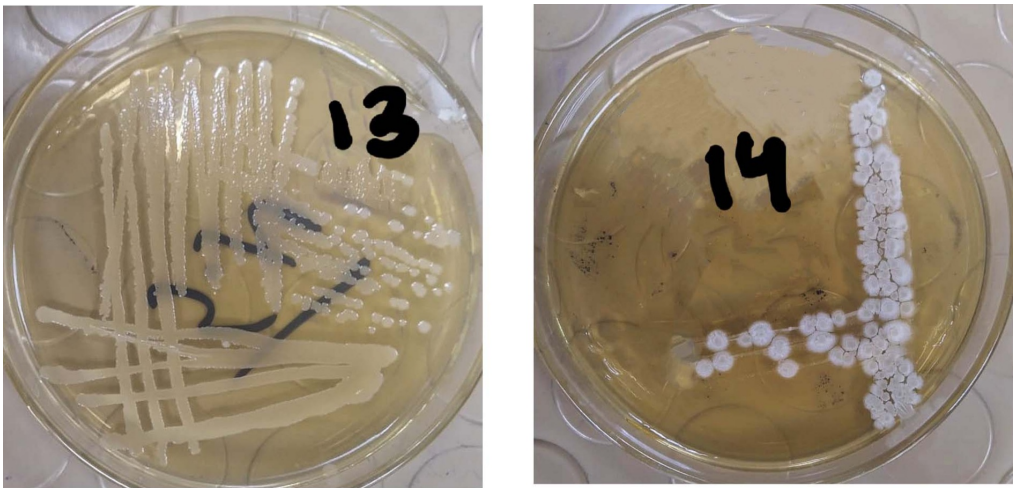


FIGURE 4.21: Bacterial isolates from smog sample N.S13 and N.S14

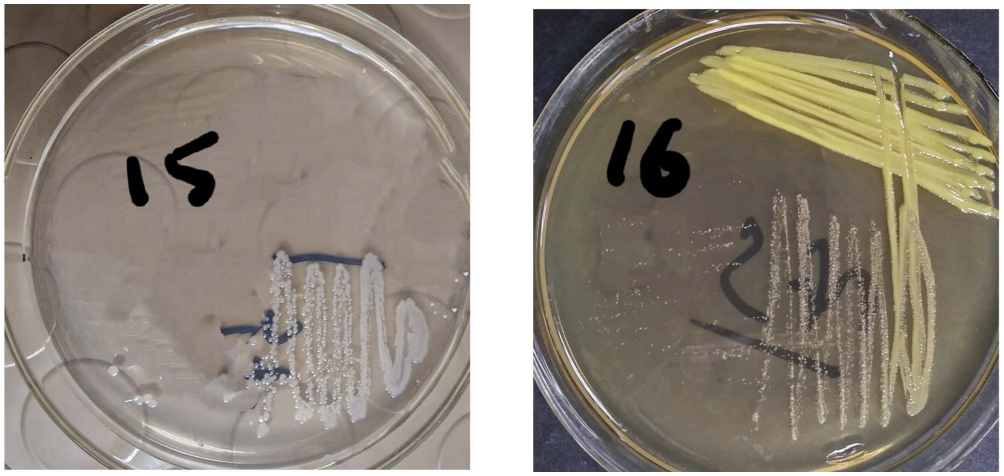


FIGURE 4.22: Bacterial isolates from smog sample N.S15 and N.S16

FIGURE 4.23: Bacterial isolates from smog sample N.S17 and N.S18

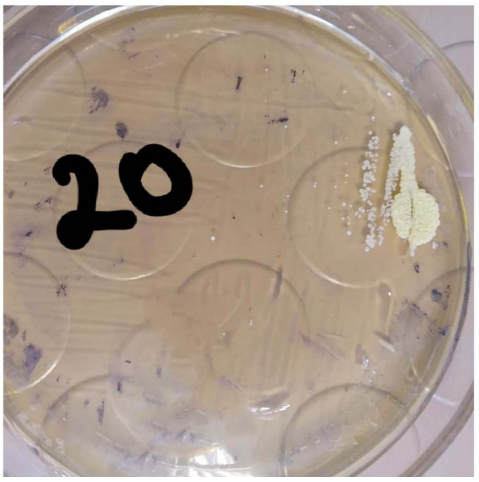
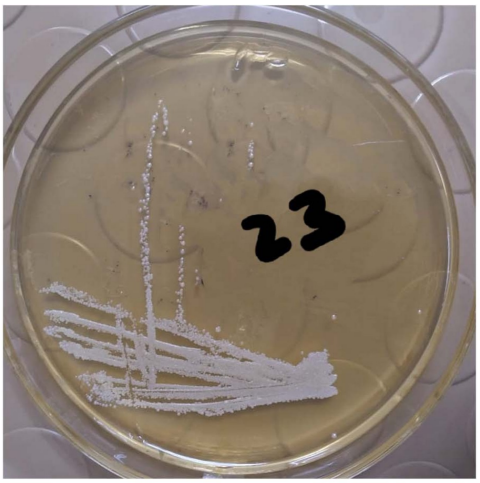


FIGURE 4.24: Bacterial isolates from smog sample N.S19 and N.S20

FIGURE 4.25: Bacterial isolates from smog sample N.S21 and N.S22



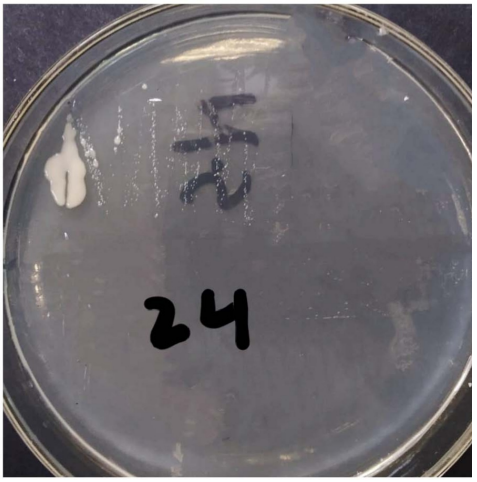


FIGURE 4.26: Bacterial isolates from smog sample N.S23 and N.S24

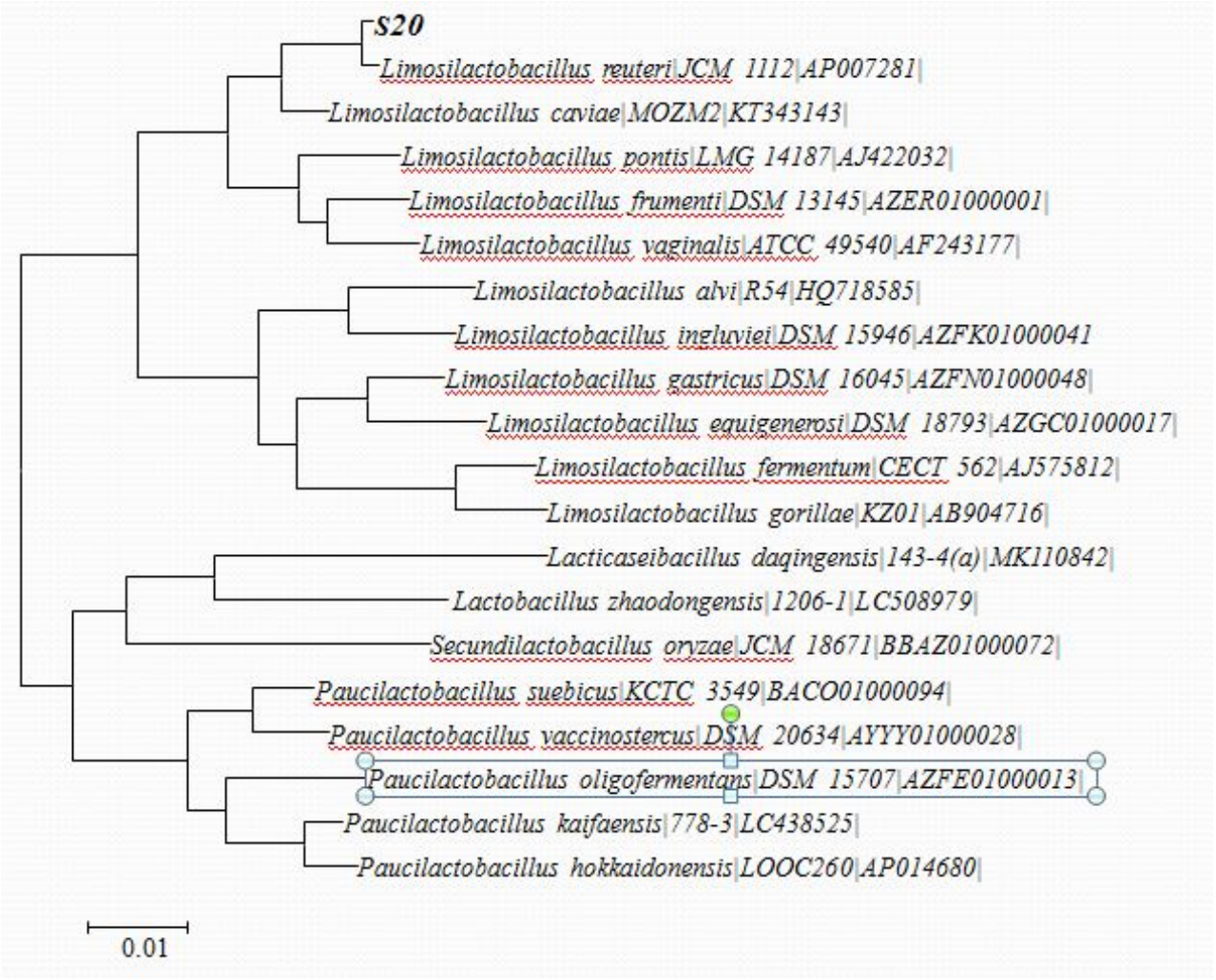


FIGURE 4.27: Evolutionary relationships of taxa By using the method of Neighbor-Joining we have made the evolutionary history of the tax [1]. The optimal sum total of lengths of all the branches is 0.36216677.

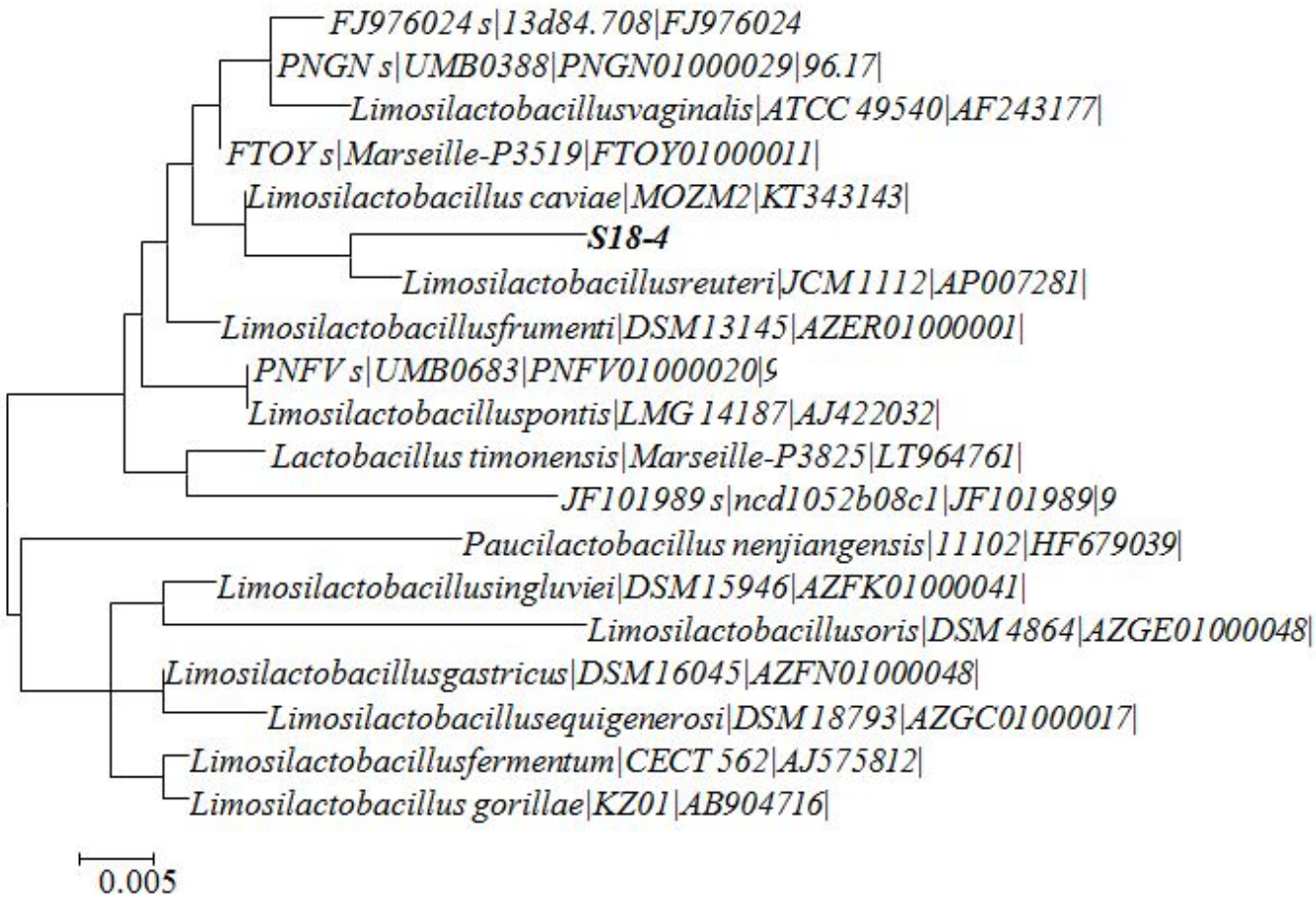


FIGURE 4.28: Evolutionary relationships of taxa By using the method of Neighbor-Joining we have made the evolutionary history of the tax [1]. The optimal sum total of lengths of all the branches is 0.36216677.

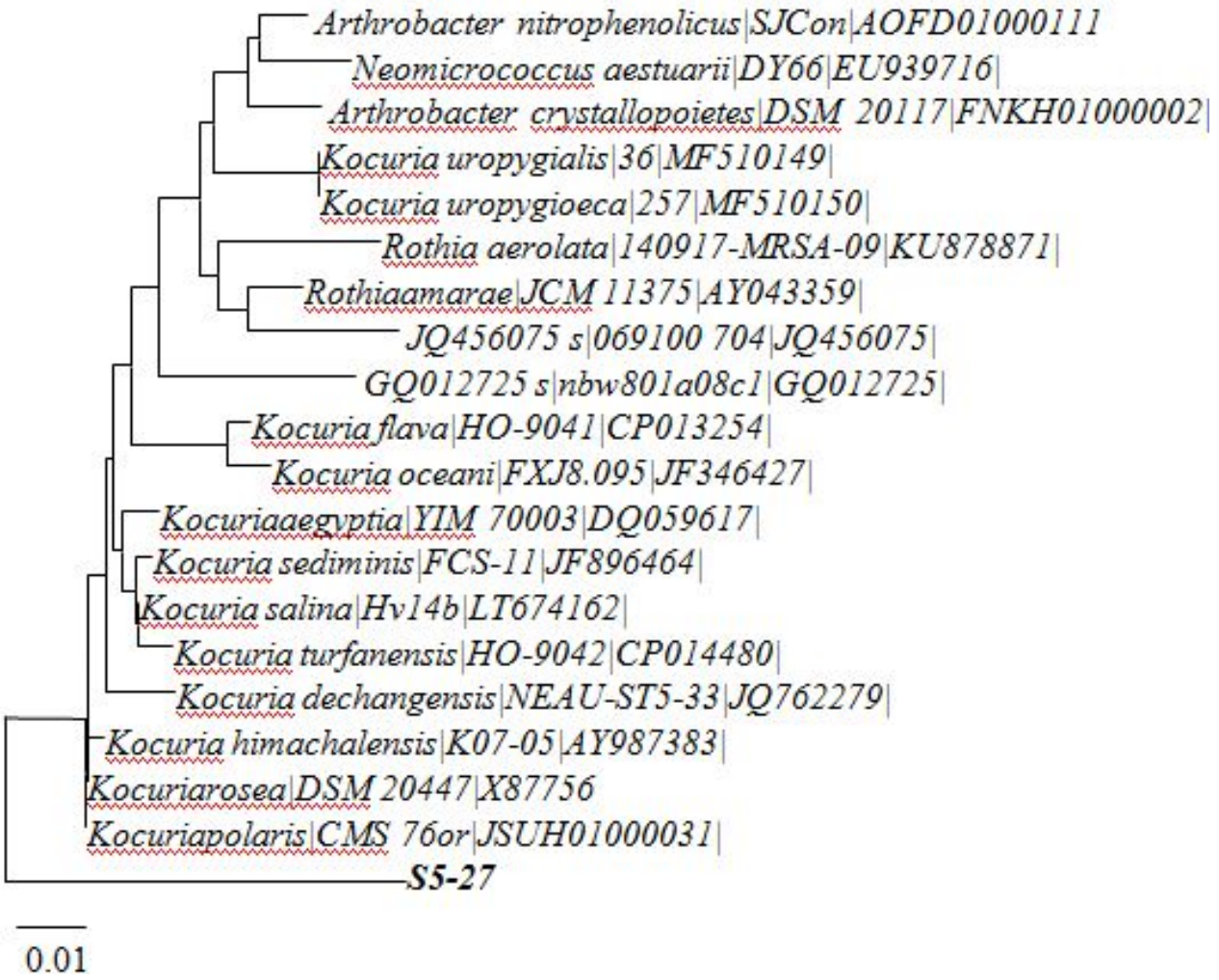


FIGURE 4.29: By using the method of Neighbor-Joining we have made the evolutionary history of the tax [1]. The optimal sum total of lengths of all the branches is 0.36216677.

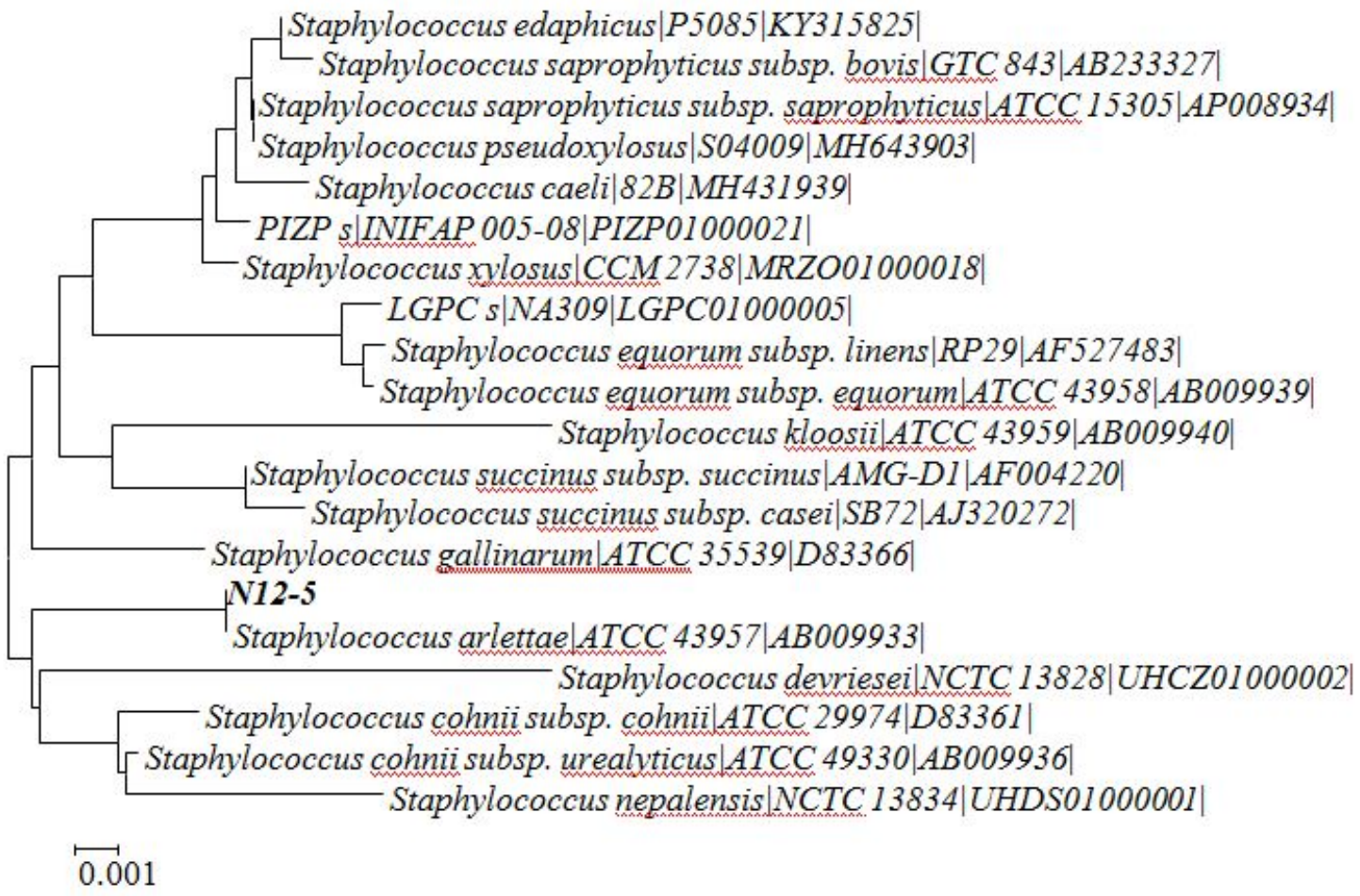


FIGURE 4.30: By using the method of Neighbor-Joining we have made the evolutionary history of the tax [1]. The optimal sum total of lengths of all the branches is 0.36216677.

FIGURE 4.31: By using the method of Neighbor-Joining we have made the evolutionary history of the tax [1]. The optimal sum total of lengths of all the branches is 0.36216677.

Table 4.2: bacterial isolates from non-smog sample

Name	Color	Shape	Elevation	Growth	Temperature
N.S1	Light yellow shiny	Round	Slightly raised	Fast	25
N.S2	Off White	Wavy	Close to surface	Fast	37
N.S3	pink	Round pointed tip	Raised	Medium	25
N.S4	Brown	Round small	Slightly raised	slow	37
N.S5	Brown	Round medium	Close to surface	slow	25
N.S6	White	Round large	Close to surface	Fast	37
N.S7	Off white	Round medium	Slightly Raised	Medium	42
N.S8	White	Irregular shape	Raised	Fast	25
N.S9	White Shiny	oval large	raised	Fast	37
N.S10	off white	Round medium	Closs to surface	medium	37
N.S11	white opaque	Round large	Slightly raised	fast	25
N.S12	white opaquet	Round small	Close to surface	slow	37
N.S13	off white opaquet	oval large	Slightly raised	fast	25
N.S14	white opaque	Round large	raised	fast	25
N.S15	off White bright	Round medium	Closs to surface	slow	42
N.S16	Yellow bright	Round small	Closs to surface	fast	37

N.S17	white bright	round	Raised	Medium	42
N.S18	off White opaque	Round small	Raised	fast	37
N.S19	off white	Round small	slightly raised	Medium	37
N.S20	light yellow	round small	raised	slow	25
N.S21	White opaque	Round small	closs to surface	medium	37
N.S22	off White opaque	wavy medium	Slightly raised	fast	37
N.S23	White opaque	Round medium	raised	Medium	25
N.S24	White	round small pointed	Closs to surface	slow	37
N.S25	White bright	oval medium	Slightly raised	fast	25

4.3 Antibiotic Susceptibility Test Results

We have 52 bacterial isolates, 25 from non-smog sample and 27 from smog sample. We find out antibiotics resistance of all bacterial isolates by disc diffusion method. We have detected MDR bacteria in both smog and non-smog samples. In our study 6/27 that makes 22.22 percent, bacteria from smog sample and 5/25 that makes 20 percent, bacteria from non-smog sample, were resistant to three antibiotic classes. Beside this 11.12 percent (3/27) bacterial isolates were found to resistant against four classes of antibiotics in smog sample, whereas in non- smog sample, 8 percent (2/25) bacterial isolates were found to resistant against four classes of antibiotics. We also found one bacterial isolate from smog and one bacterial isolate from non-smog samples that were resistant against five antibiotic classes except that of vancomycin. Five most resistant strains from both samples were identified through genome sequencing. These strains were identified as *Lactobacillus reuteri* which are basically anaerobic Gram-positive, rods which are heterofermentatic lactic acid producing bacteria that are naturally occurring in the gut of many organisms, like humans, chickens and mic. They are also present in the breast milk of human and they are also present in biofilms. These bacteria have pro-biotic characteristics there, when taken in good amount can induce health benefits. These bacteria live in gut of many animals where they are constantly been exposed to various antibiotics. Therefore these bacteria do have acquired antibiotic resistance in part of struggle for their survival. A previous study has shown that VLactobacillus reuteri has shown antibiotics resistance to various antibiotics [5]. So it is shown in our studies that our strain is also resistant to various antibiotics that are in accordance to previous studies. *Staphylococcus arlettae* is our second resistant strain that is basically a gram-positive bacterium usually isolated from skin of birds and mammals. It is also found in industrial effluents of textile factories were they are able to degrade different dyes like azo dyes [2].Staphylococcus arlettae although is normally considered as a commensal bacteria, it has shown associated with different types of infections or in those environments where a large amount of antibiotics is applied.

TABLE 4.3: bacterial isolates from smog sample

S.Name	Ampicillin	Tetracycline	Erythromycine	Nalidixic Acid	Imipenem	Vancomycin
s1	Resistant	Resistant	Resistant	Resistant	Resistant	Non-Resistant
s2	Resistant	Resistant	Resistant	Resistant	Non-Resistant	Mild Resistant
s3	Resistant	Non-Resistant	Resistant	Resistant	Resistant	Non-Resistant
n1	Resistant	Resistant	Resistant	Resistant	Resistant	Non-Resistant
n2	Non-Resistant	Resistant	Resistant	Resistant	Non-Resistant	Resistant

Therefore resistance is quite obvious in these bacteria. A previous study showed that *Staphylococcus arlettae* is resistant to all -lactams including ampicillin and imipenem. Resistance to other antibiotics has also been shown, for example erythromycin, fusidic acid, vancomycin and tetracycline [3]. Our third strain showed 90 percent similarity to *Kocuria sediminis*, which is a gram-positive bacterium that was first isolated from a sediment samples that were taken from Kerala, India. This is an actinobacteria that produces antibiotics themselves. Actinobacteria are usually resistant against most of antibiotics, so the resistance shown by our stain is in according to previous studies. Our fourth bacterial specie belongs to *Burkholderiales* which are Proteobacteria. These are Gram-negative bacteria and also includes pathogenic bacteria like *Burkholderia, Ralstonia and Bordetella. Burkhulderiales* bacteria are the commonly found in soil and water.

These are basically pathogenic bacteria of human and plants and cause serious lungs infections and cystic fibrosis [4]. These bacteria have resistance against many antibiotics and along with *Staphylococcus aurous* they are involve in causing complications in antibiotics practices. Most of these sequenced strains 4/5 belong to Gram positive bacteria, while only one was Gram negative. It can be said that Gram positive bacteria has dominated the bacterial community on air particulate matter both in smog and non-smog samples. All of these strains have their specific habitat, ie; *Lactobacillus reuteri* are present in gut of animals while Staphylococcus arlettae is found in skin of birds and mammals. Similarly *Kocuria sediminis* is present in bottom sediments of ocean and *Burkholderiales* are present in soil and water.

Presence of these bacteria on air particulate matter indicates that air particulate matter is a fertile place for the bacteria to inhibit. Bacteria uses different routes to adhere themselves to the air particulate matter. Anthropogenic activities will definitely enhance the ability and chance of bacteria to be aerosolized on air particulate matter.It is known previously those resistant bacteria which are present in different environments, like fecal bacteria, can transmit their antibiotic resistance genes to other bacteria which are non-resistant. Similarly it is expected that

resistant bacteria in air also can transmit their resistant genes to indigenous non-resistant microbes inside the human body when they are taken in with breathing. Enrichment in antibiotic-resistant bacteria is promoted by the presence of antimicrobials in the environment. Bacterial populations in air particulate matter consist of three types of bacteria. First, antibiotic producing bacteria second bacteria which are resistant to several antibiotics third those bacteria which are non resistant to antibiotics. First two groups are naturally carrying resistance genes for antibiotics. By considering these factors together, air particulate matter can be considered as reactor system, where resistant genes can spread from these two groups to third group of bacteria which are not resistant to antibiotics.

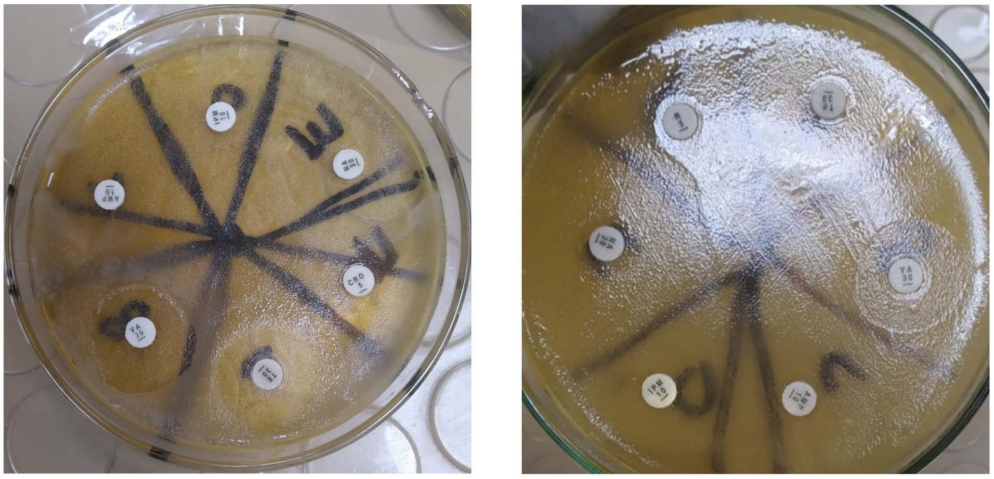


FIGURE 4.32: left:N12 Staphylococcus arlettae; Right:N4 Burkhulderiales

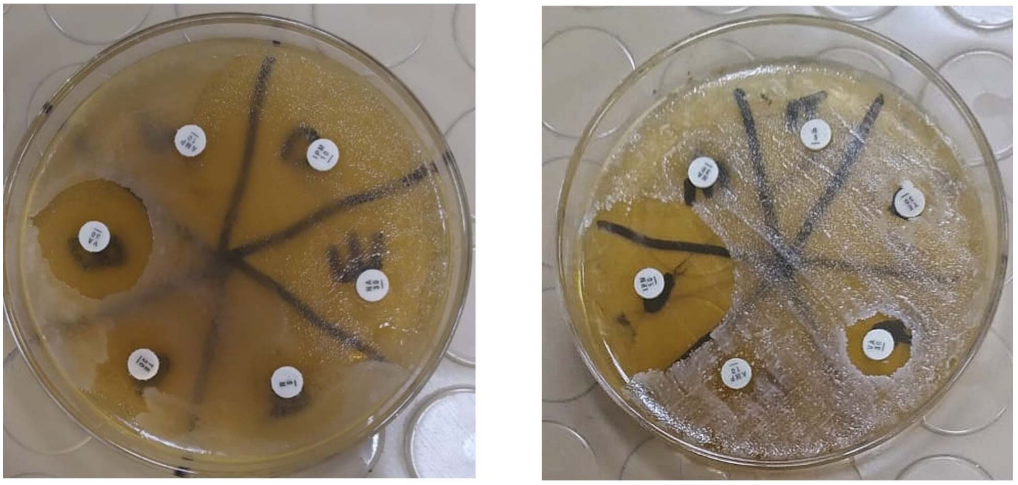


FIGURE 4.33: left:S20 Limosilactbascillus reuteri; Right:S18 Lactobassilus reuteri

Under these circumstances, multidrug resistance characteristic bacteria may also arise so easily, and it may also transfer resistant genes to pathogenic bacteria which are present on air particulate matter, as it occurs in other environments. Hence it may lead pathogenic bacteria to become multi-drug resistant. Finding

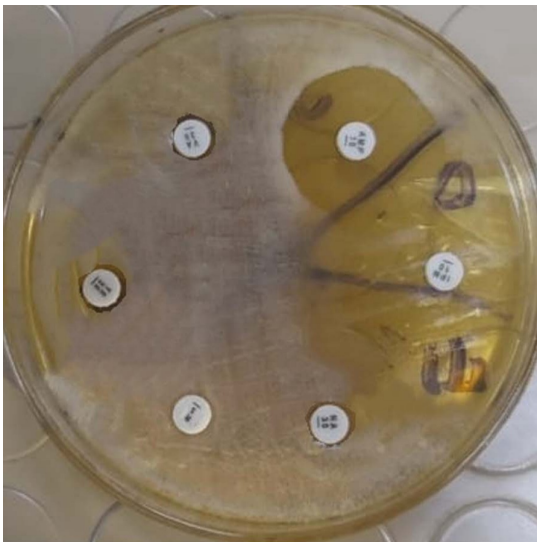


FIGURE 4.34: S5:Kocuria sediminis

of these bacteria indicates that the ARGs-carrying bacteria can be transferred from soil, water, skin of mammals, fecal materials and many other habitats, to air particulate matter by human activities like industrial soot formation, dust production, agricultural practices, and automobile exhaust and by formation of secondary aerosol particles. In the air these resistant bacteria can travel long distances by wind circulation and can contaminate for away those environments which do not have resistant bacteria previously.

4.4 Results of Antibiotics Resistance Genes in Air Particulate Matter

In our study we have extracted DNA from particulate matter in air and perform PCR to amplify six antibiotics resistance genes to check their presence on air particulate matter. Out of these six genes we have detected four only three genes in our smog sample. Detected genes are blaTEM, qnrA and intl1. It is probably

the first study that is carried out to detect the antibiotics resistance bacteria and antibiotics resistance genes on air particulate matter in Pakistan. As we have mention earlier that four genes in this study are consider as indicator genes for antibiotic resistance. Out of these four genes we have detected two resistance genes on air particulate matter of Islamabad during smog days.The size of PCR products were analyzed in relation with the size of ladder. In our results we have find three distinct bands that confirmed the presence of relevant ARGs. These bands are formed for blaTEM, qnrA and Intl1 genes. The product of these genes has sizes of 450 bp, 515bp and 290 bp pairs for blaTEM, qnrA and Intl1 genes. We do not detected any bands for tetM, vanA, and sul3 genes. out of these three detected genes blaTEM and intl1 gene are known as indicator genes.

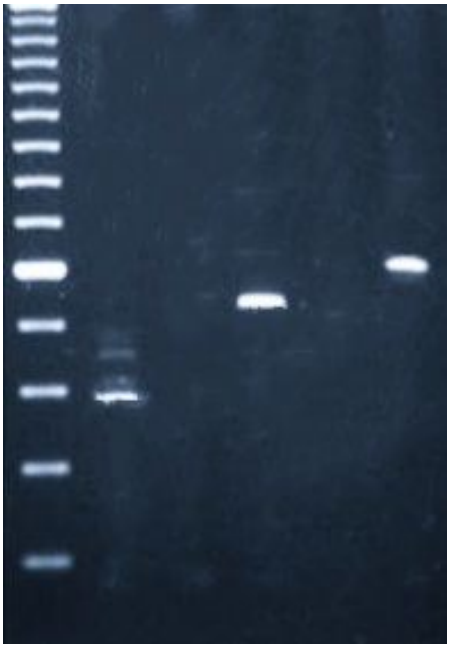


FIGURE 4.35: three distinct bands are shown: on the left a band of 290 bps indicates the pressence of intl1 gene: in middle a band of 450 bps indicates the blaTEM gene: on right a band of 515 bps indicates the presence of qnrA gene

blaTEM gene provides resistance to bacteria against Beta-lactum antibiotics which includes second and third generation of cephalosporins like cefotaxime ceftriaxone and ceftazidime. These antibiotics are basically penicillin derivatives. Gene blaTEM is involve in production of special enzymes called beta-lactamases which are affective against break down of -lactam ring that is present in penicillin. In the presence of blaTEM gene bacteria acquire Extended spectrum beta-lactamases

(ESBL) enzymes which hydrolyze and breakdown penicillins and aztreonam so that these antibiotics become les affective in the treatment of bacterial infections. (Bush and Jacoby, 2010). When we compare the cost of treatment, it is most obviously observed that patients having infections due to ESBL producing bacteria are usually have a poor outcome of antibiotic treatment that results in increased cost. While on the other hand those patients which are infected with non-ESBL-producing bacteria outcome with less of their cost. Beside the cost, patients have to stay for longer time in the hospital for the treatment due to ESBL producing bacterial infections. BlaTEM gene is usually present on the highly transferable plasmids. Hence these genes can easily be transferred from one bacterial stain to other causeing the phenomenon of multi-drug resistance more widespread.

In our study blaTEM gene is detected on air particulate matter. This gene is usually encoded by large plasmids (100kb) that are transferable from strain to strain of same specie and strains of different bacterial species. Gene blaTEM is wide spread in natural environments. However; recently it was also detected on the air particulate matter of smog in Beijing [65]. This gene is also detected in the air particulate matter in different cities all around the world with relatively high abundance is observed for San Francisco. On the other hand, Johannesburg, Zurich and Hong Kong had the lowest relative abundances of b-lactam resistance genes on air particulate matter [66]. As it is show in our study that blaTEM gene is present on air particulate matter in Pakistan, a similar resistance pattern is also shown in previous study about the bacterial pathogens, causing hospital acquired infections in the community of Pakistan[64]. n this study bacterial pathogens were shown to have resistance against b-lactum antibiotics and presence of Bla tem in our study is in accordance with this study.

We have detected the class 1 integron gene intl1 in our sample. The conditions for reaction were as follow, initial denaturation is done at 94°C that take 4 min then 10 cycles are performed for denaturation at 94°C for 1 min, annealing was done at 62–53°C for 30 s and extension is done at 72°C for 2 min, Horizontal gene transfer is a major phenomenon in antibiotics resistance dissemination. Intigrons along with transposons and plasmids makes major contribution in horizontal gene transfer.

Integraons are of special interest because they can load different resistant genes within them. Intigron is basically composed of three regions: first 5 conserved sequence (5CS), second is a gene cassettes that is internal variable part of intigron that contains one or more antibiotics resistance gene and third part is 3 conserved sequence (3CS). The 5CS region contains a gene called as integrase gene (intI). This gene produces an integrase enzyme (recombinase) which is responsible for insertion of resistance-gene cassette. A promoter (Pc) is also present in 5CS, which is responsible for expression of gene cassettes. The 3CS contains a compound (qacE-1) and resistance gene (sul1) and an open reading frame is also present which have unknown function. There are five classes of integrons have been discovered uptill now. These are different from one another at intI sequences level. Class 1, 2, and 3 integrons are mostly common and are found in many bacteria while 4 and 5 integrons classes are relatively rare. It is a widely distributed in different environments.

Uptill now this gene has been detected in multiple environments which includes human microbiota, animal-associated environments like cattle feed yards, water, soil, sediments, mine, wastewater or sludge, hospital environments , and ambient particulate matter.8 in a recent study it was indicated that IntI1 gene might be playing a diluting role for the horizontal gene transfer across the different land gradients, for example, urban to rural area and from rural to industrial areas[68]. In a previous study in which global survey for antibiotics resistance genes on air particulate matter was performed, IntI1 gene was found to present all nineteen cities of world. It shows their wide spread presence in nature. Clinically Class 1 integron gene is the most frequent type of mobile genetic element that is present in gram negative bacteria. Initially the presence of this gene was only shown in gram negative bacteria ;however now it is clear that it is also present in gram positive bacteria , and most of the reported antibiotic-resistance gene cassettes are found in this class[67].

Quinolones are the antibiotics which are basically used to treat Gram-negative bacterial infections of Enterobacteriaceae and others. These are synthetic drugs and Fluoroquinolones are their derivatives which are considered as broad-spectrum

antibiotics. Quinolones are extensively used antibiotics all around the world for the treatment of various bacterial infectious diseases so resistance against these drugs was not a big surprise. There are three mechanisms through which quinolones resistance can occur, (1) mutations of those genes which targets the DNA gyrase enzyme and as well as topoisomerase IV enzyme; (2) change in the efflux pumps activity so that it decrease the internal concentration of quinolones in the cell, (iii) the acquisition of resistance genes that are present on plasmids. After the discovery of plasmid mediated quinolone antibiotics resistance genes, researchers have started to discover different alleles of plasmid mediated quinolone antibiotics resistance genes. A large number of qnr alleles have been discovered on plasmids and in bacterial chromosome. Up till now About 100 qnr genes variant have been described mainly from Enterobacteriaceae, and these genes are grouped into five distinct families: qnrA, qnrB, qnrC, qnrD and qnrS.

Presence of these resistance genes with intl1 is very alarming. It indicates that the resistance genes can easily be transfer to other bacteria with the help of intl1. In fact intl 1 gene provides an easy acquisition mode to bacteria for these resistance genes to enter into bacteria through HGT. It is described earlier tha resistance gene sul1 resided in conserved region towards one terminal. Class 1 integron is a mobile genetic element that is capable of carrying various other ARGs along with sul1 and capable of horizontally transfer these resistance genes among other bacterial cells in the environment. So the presence of blaTEM and tetM along with intl1 gene shows that these two resistance genes would easily be transmitted in bacterial community of smog particulate matter in Islamabad. It is previously established that bacterial community show resistance against those antibiotics whose genes are frequently present in the environment.

As we have detected blaTEM and qnrA genes with intl gene, it was expected that bacterial community of smog particulate matter would be resistant to these to antibiotics. Our antibiotics susceptibility test also indicated the pattern, where most of bacteria were resistant to these antibiotics. Drug consumption data shows an important aspect in this regard. First we look the global patterns of antibiotics use and presence of antibiotics resistance genes on air particulate matter, than we

compare it to Pakistan. It is reasonable to conclude from global data that the antibiotic use patterns for different antibiotic types are responsible for the variations in the relative abundance and distribution of ARGs types on air particulate matter. By reanalyzing the existing global antibiotic consumption data we founds that five major common used antibiotics includs -lactams, quinolones, macrolides, tetracyclines, and aminoglycosides. In previous study it was shown that there is a positive linear correlation between the global antibiotic drug consumption in hospitals and relative abundances of airborne ARGs. This correlation is shown in the fig below.

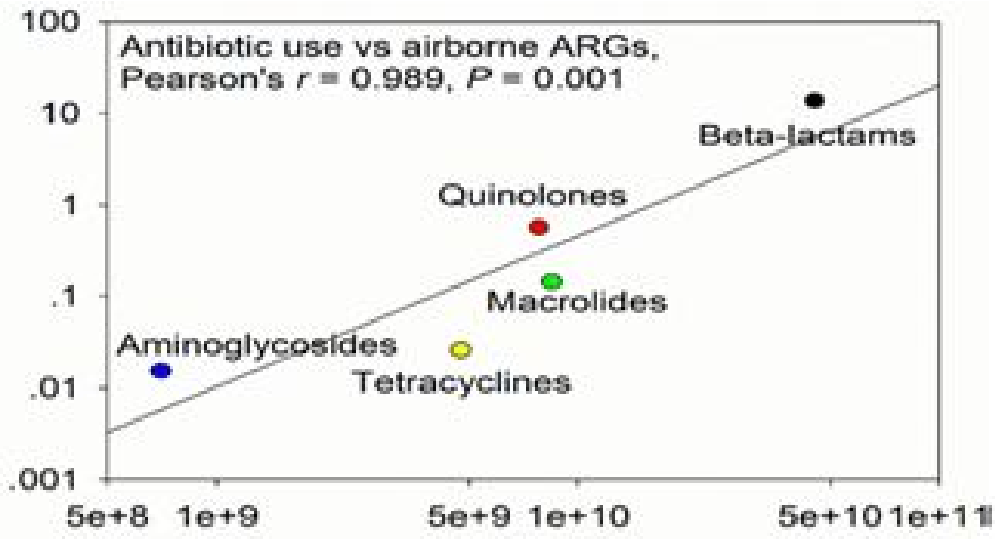


FIGURE 4.36: correlation of the relative abundances of airborne ARG types in the present study with the global antibiotic consumption in 2010 reported previously35 in five common antibiotic types.

Similar trends in consumption of antibiotics can be seen in Pakistan. Of all antibiotics, the most widely used antibiotics are coamoxiclav which is a beta-lactum, ciprofloxacin and amoxicillin. So the top most class of antibiotics used are beta lactum as it is used worldwide[69]. It can be interpret that airborne bacteria on particulate matter are more dangerous than individual genes. Although these genes present on particulate matter but the acquisition of these genes requires a complete system of acquisition. This complete system is present in living bacteria. So the living bacteria are more dangerous than the genes itself. However long term affects of presence of these resistance genes on particulate matter is needed to be explore. In this study we just only highlight the threat of airborne ARGs

which are present on air particulate matter during the smog and emphasize that current air quality standards must be reviewed in terms of prevalence of ARGs on particulate matter and public health. Environmental hotspots like smog, where ARBs are abundant or the transfer of ARGs is promoted, are critical points for resistance control. Good examples of such critical points are characterized by a high prevalence of resistance or by the occurrence of resistance determinants of emerging concern. These locations comprise habitats that are influenced by human activities (e.g. wastewater from animal husbandry and intensive food production facilities). Sites subjected to frequent discharge of antibiotic residues have been shown to be potential hotspots for the proliferation and spread of new resistance determinants to human and pathogenic bacteria and should be considered as critical control points. Although some of the antimicrobials administered to animals are used exclusively in veterinary applications, most belong to the same structural families that are used in human medicine. As they share the same basic chemical molecular structures and mechanisms of action, these antibiotics are assumed to put selective pressures on human commensal and pathogenic bacteria. Large quantities of antibiotics that are administered to animals in intensive production sites are discharged, often unmetabolized with manure and slurry when applied as fertilizer, and thus contaminate soils as well as surface water and groundwater. At present, it is difficult to ascertain whether antibiotics reaching the environment at low concentrations exert a substantial selective pressure on ARGs.

Urban, hospital and pharmaceutical industry wastewater is among the main sources of antibiotic contamination in soil ecosystems. In the environment, these contaminants can reach water resources for drinking water production, enter the food chain or reach clinically relevant niches. These effects can be potentially even more pronounced when irrigation with wastewater effluents (wastewater reuse schemes) is applied. Water reuse is already a common practice in many regions of the world owing to increased water scarcity, mainly in arid and semiarid regions. Most of the wastewater treatment plants worldwide, in particular those using mechanical and biological treatments, are primarily designed to remove organic compounds, nutrients (e.g. nitrogen and phosphorous) and suspended solids. However, the

currently available wastewater treatment processes have limited capability to efficiently remove organic micropollutants, including antibiotics and other antimicrobial agents. Similarly, certain ARGs can survive the wastewater treatment processes with a maintenance (or even an increase) of resistance prevalence compared to the pretreatment levels. These features require the immediate implementation of technological solutions capable of mitigating ARB and ARGs in wastewater to safe levels. Although the definition of a "safe level" may be difficult to achieve, it is at least necessary to find an agreement on the threshold values below which the probability of significant proliferation of an ARG is severely impaired.

Chapter 5

Conclusions and Recommendations

As ARGs are immerging biological pollutants of air, much is needed to be exploring about the health risks associated with the presence of these resistance on air particulate matter. When these ARGs are inhaled with the air they can disrupt the equilibrium of bacterial community present in our lungs. These genes can produce resistance in micobiota of our lungs. Conditions can be goes wrong when a person is going through antibiotic treatment. As we know these antibiotics creates a selective pressure on the bacteria and bacteria are looking for such resistance genes. Under such conditions of selective pressure of antibiotic treatment these resistant genes will easily be disseminate to the microbial community of our body. It has long term consequences.

Those bacteria which are not pathogenic, when acquire these genes become resistant. So, microbiota of our body is becoming resistant more and more and may become a reservoir of resistance. When a pathogenic bacteria will enter to our body it will easily take resistance genes from our natural bacteria of our body. It may also affect the immune responses of our body and may disrupt the balance of

our immune system that could be result in over activation of immune response. Although ARGs are creating multiple health problems However; the most dangerous risks of these resistance genes are severe respiratory diseases.

As these ARGs are present on air particulate matter that can travel long distances with wind circulations so the other place where antibiotic resistance is not present can be affected with second hand exposure of ARGs. As the anthropogenic activities are more in urban area, it is expected that more ARGs are loaded to particulate matter from urban cities. Hence urbanization is involved in another global problem of antibiotics spread.

We have observed in this study that there is a co-relation between use of antibiotics antibiotics resistance bacteria and antibiotics resistance genes. Those antibiotics which are used extensively scince last many years, resistance is also persists for those antibiotics. As we noticed that b-lactums and quinolones are major antibiotics which are used more extensively in previous years therefore bacteria are frequently found in natural environment which are resistant to these antibiotics. Similarly we have detected blaTEM and qnrA genes which are responsible for providing resistance to those antibiotic. Misuse of antibiotics has accelerated the spread of these genes in bacterial communities.

Misused antibiotic when releases into natural environment they cause a selective pressure on bacteria. Bacteria will try to acquire resistance genes from their soundings and from other bacteria to combat these circumstances. Bacteria having large populations and better contact rate will be favored and naturally selected. Other bacteria will not survive hence the bacterial community is shifting from non- resistant to resistant as a whole.

In-fact antibiotics dissemination is an example of mendal,s law of natural selection. Those resistant genes that have more benefits for bacteria with less cost of fitness are likely to spread at faster rate than the others. So there are two outputs of our study: we should control those bacteria which have larger populations and better contact rates and work against those resistant genes which provides less fitness cost. Environmental hotspot like particulate matter, gut microbiota, waste

water treatment plants and hospitals should be targeted to control the antibiotics dissemination.

5.1 Future Prospects

A better awareness of the abundance and types of airborne ARGs is critical to understanding current infectious disease transmission and providing valuable information for re-evaluating air quality assessment practice As for as in my knowledge, it is the first research in our country on this topic. It is a pilot study that will open a gate for the researcher to investigate the presence of ARGs and ARBs on PM in different cities across the country during the smog and non smog days.

Furthermore, by expanding this work we will be able to identify the sites (urban ruler, agricultural, industrial or clinical) which are considered as hotspot for emission of PM that is loaded with ARGs and ARBs.

In this research study we have discuss the potential role of air particulate matter in dissemination of antibiotic resistance on a very small scale. Detail studies are required to assess the complete role of smog and air particulate matter in dissemination of antibiotics resistance.

Pakistan Lahore and southern Punjab are severely affected by smog. This study can expand to those areas where smog conditions are worst, to assess the severity of the problem.

Beside this there is need of investigation on spatial temporal variation in the concentrations and relative abundance of ARGs and ARBs in air particulate matter.

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