IDENTIFICATION AND ANALYSES OF ANTIBIOTIC RESISTANT STRAINS FROM ENVIRONMENTAL EFFLUENTS; VIRTUAL SCREENING OF NATURAL COMPOUNDS TO PROPOSE POTENT LEADS

Thesis submitted to the Degree of Doctor of Philosophy (Science)

In

Microbiology

By

Meesha Singh

Post Graduate and Research Department of Microbiology

St. Xavier's College (Autonomous), Kolkata

Registration No.: PHD/18/MCB/06

Affiliated to the University of Calcutta

2023

ACKNOWLEDGEMENTS

Firstly, I would like to express my deepest gratitude and appreciation to my supervisor, Dr. Mahashweta Mitra Ghosh for her unwavering support and invaluable guidance throughout my doctoral journey. Her expertise, encouragement, and constructive feedback have played a pivotal role in shaping the direction and outcome of this research.

I would like to thank my Co-supervisor Dr. Sayak Ganguli for his continuous guidance, patience, and mentorship. His constructive criticism, valuable suggestions and innovative ideas have really helped me to shape up my thesis.

Both of my mentors have always motivated me and been my guiding force since the beginning. Throughout this journey I faced a lot of obstacles but my mentors did not give up on me and kept on pushing me to complete this venture.

I would like to sincerely thank Rev. Dr. Dominic Savio, S.J. (Principal) for providing a conducive academic environment and access to resources necessary for conducting my research. I am grateful to Prof. Bertram da Silva, Vice-Principal (Arts and Science) for his support. I am thankful to Dr. Tapati Dutta, Dean of Science for her contributions.

I would like to express my gratitude to Dr. Samrat Roy, Ph. D. Coordinator for his kind support during my PhD tenure.

I am immensely grateful to Dr. Subhra Kanti Mukhopadhyay, Associate Professor, Dept. of Microbiology, Burdwan University; Dr. Maitree Bhattacharyya Professor; Director, Jagadis Bose National Science Talent Search, Kolkata, and all other members of my PhD committee for their insightful comments, suggestions, and contributions. Their expertise and critical evaluation have immensely enriched the quality of this thesis.

I owe my deep gratitude to Dr. Arup Kr. Mitra (Founder Head and Controller of Examinations). He has played a pivotal role in my Ph.D. tenure from the very beginning. I am sincerely thankful to Dr. Sudeshna Shyam Chowdhury (HOD, Department of Microbiology) for her positive affirmations and guidance.

I express my gratitude to all the faculty members of Department of Microbiology, St. Xavier's College, Kolkata, viz. Dr. Kasturi Sarkar, Dr. Madhumita Maitra, Dr. Anindita Banerjee, Prof. Debjani Dutta, Dr. Jaydip Ghosh, Dr. Riddhi Majumder and Prof. Lopamudra Mitra for their immense support and valuable advices that I have received throughout my Ph.D. tenure.

I would like to sincerely thank Dr. Chinmay Saha and Dr. Upal Das Ghosh for their kind help and guidance.

I am indebted to Souradip Basu, Rupsha Karmakar, Kaustav Mondal and all other colleagues and fellow researchers for their camaraderie, stimulating discussions, and willingness to collaborate. Their support and friendship have made this journey more enjoyable and rewarding.

I am grateful to West Bengal DST for financial support which allowed me to focus on my research with greater dedication.

I am immensely grateful to the laboratory technicians, Mr. Pintoo Biswas, Mr. Debashish Das, Mr. Bittu Baidya and Mr. Biswanath Sen for their help and cooperation.

Lastly, I would like to express my heartfelt appreciation to my parents, my husband and my daughter Navya for their unwavering love, encouragement, and understanding throughout this challenging endeavour. Last but not the least I would like to thank Almighty God for giving me strength and courage to complete this journey.

This thesis would not have been possible without the collective contributions and support of all these individuals and institutions. I am truly grateful for their impact on my academic journey and the completion of this research.

-Meesha Singh

List of Abbreviations

AR	Antibiotic resistance
AMR	Antimicrobial resistance
MRSA	Methicillin-resistant *Staphylococcus aureus*
ESBLs	Extended-spectrum beta-lactamases
ARGs	Antibiotic resistance genes
WWTPs	Wastewater treatment plants
MRGs	Metal resistance genes
BRGs	Biocide resistance genes
HGT	Horizontal gene transfer
ARB	Antibiotic-resistant bacteria
MDR	Multi-drug resistant
XDR	Extensively drug resistant
STPs	Sewage treatment plants
pH	Negative of log of hydrogen ion concentration
CFUs	Colony-forming units
NGS	Next-generation DNA sequencing
PDAs	Plant-derived antimicrobials
WHO	World Health Organization
EUCAST	European Committee on Antimicrobial Susceptibility Testing
CLSI	Clinical and Laboratory Standards Institute
PCR	Polymerase chain reaction

°C	Degree Celsius
%	Percentage
µg	Microgram
µl	Microliter
µM	Micromole
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
dNTP	Deoxynucleotide triphosphate
g	Gram
mg	Milligram
ml	Milliliter
rDNA	Recombinant DNA
Mb	Megabyte
pM	Picomole
bp	Base pairs
OTUs	Operational Taxonomic Units
rpm	Rotation per minute
TAE	Tris –Acetate-EDTA
DEG	Differentially Expressed Genes

SERIAL NUMBER	Contents	PAGE NUMBER
1.	**Abstract**	1-2
2.	**1. Introduction**	3-19
	1.1 Emergence of antibiotic resistance	3-5
	1.2 Role of Wastewater in the Dissemination of antibiotic resistance in the Environment	5-9
	1.2.1 Antibiotic-resistant Bacteria and Genes in Sewage and Hospital Wastewater	7-8
	1.2.2 Antibiotic-resistant Bacteria and Genes in Rivers	8-9
	1.2.3 Antibiotic-resistant Bacteria and Genes in Surface Water and Groundwater	9
	1.3 Environmental Surveillance of antibiotic resistance	9-17
	1.3.1 Culture-Based Methods	10-13
	1.3.1.1 Importance of culture media for growth and purification of bacteria	10-11
	1.3.1.2 Characterization of bacteria	12
	1.3.1.3 Morphological Study of Bacteria	12-13
	1.3.1.4 Role of Culture-based Methods in AMR Surveillance	13
	1.3.2 Community Genomics-Based Methods	13-17
	1.3.2.1 Importance of 16S rRNA gene	14-15
	1.3.2.2 Databases used	15
	1.3.2.3 Role of Molecular-based Methods in AMR Surveillance	16
	1.3.3 Metagenomics-based methods	16-17
	1.4 Plant-derived antimicrobials for therapeutic purposes	
3.	**2. Literature Review**	20-50

	2.1 Antibiotic Resistance as an environmental problem	20-26
	2.1.1 Bacteria level	21
	2.1.2 Individual human level	21
	2.1.3 Population level	21
	2.1.4 The Environmental Resistome	22-26
	2.2 Classes of Antibiotics and subsequent resistance development in Pathogens	27-28
	2.2.1 Antibiotic Grouping by Mechanisms	28
	2.2.2 Antibiotic Grouping by Structure	28
	2.3 ARB and priority pathogens: Global and Indian Scenario	29-31
	2.3.1 WHO priority pathogens	29
	2.3.2 XDR *Escherichia coli*	29-30
	2.3.3 MDR and Pan-drug-resistant *Klebsiella pneumoniae*	30
	2.3.4 Indian scenario	31
	2.4 Methods for Antimicrobial Resistance Diagnostics	31-41
	2.4.1 Phenotypic Methods	31-32
	2.4.2 Community Genomics-Based Methods	32-33
	2.4.3 Next-generation sequencing (NGS)	33-36
	2.4.4 Metagenomics-based methods	36-41
	2.5 Computer-aided Drug Discovery	41-44
	2.5.1 Theories involved	41-42
	2.5.2 Molecular docking	42-44
	2.6 Plant-derived compounds: Potential antibacterial agents	45-50
	2.6.1 Plant metabolites	46
	2.6.2 Classes of secondary metabolites	46-47
	2.6.3 PDA: Mechanisms of action	47-48
	2.6.4 Screening of PDA for potential leads	48

	2.6.5 Antibacterial Potential of Ethnomedicinal Ferns in India	49-50
4.	**3. Objectives of the study**	51
5.	**4. Materials and Methods**	52-69
	4.1 Sample collection	52-53
	4.1.1 Study area	52-53
	4.2 Evaluation of physicochemical parameters of the sampling stations	53
	4.3 Culture-independent analysis: Metagenomics profiling	53-55
	4.3.1 Design of PCR primers with multiplexing index and Illumina sequence adapters	53
	4.3.2 PCR amplification of target region	54
	4.3.3 Sequencing	54
	4.3.4 Bioinformatics analyses	54
	4.3.5 16s rRNA Metagenome Analysis	54-55
	4.4 Taxon set enrichment analysis	55
	4.5 Pathway analysis	55-56
	4.6 Culture-dependent analysis: Microbiological characterization	56-61
	4.6.1 Pure colony isolation	56-57
	4.6.2 Biochemical characterization	57-61
	4.6.2.1 Urease Test	57
	4.6.2.2 Catalase Test	57
	4.6.2.3 Nitrate Reduction Test	57-58
	4.6.2.4 Starch Hydrolysis Test	58
	4.6.2.5 Oxidase Test	59
	4.6.2.6 Citrate utilization test	59
	4.6.2.7 Indole test	59
	4.6.2.8 Voges Proskauer Test	60
	4.6.2.9 Methyl red Test	60-61
	4.7 Antibiotic resistance profiling	61-63

	4.8 Molecular identification	63-66
	4.8.1 Isolation of genomic DNA from bacteria	63-64
	4.8.2 Agarose gel electrophoresis for visualization of genomic DNA	64
	4.8.3 Amplification of 16S rRNA gene using Polymerase chain reaction (PCR)	65
	4.8.4 PCR product purification by agarose gel electrophoresis	65-66
	4.8.5 Sequencing of purified PCR products	66
	4.9 Phylogenetic analysis	66-67
	4.10 Computational Analyses	67-69
6.	**5. Results**	70-114
	5.1 Evaluation of physicochemical parameters of the effluents	70-72
	5.2 Metagenomics profiling	72-75
	5.3 Taxonomic diversity determination	76
	5.4 Taxon set enrichment analysis	77-78
	5.5 Pathway analyses	79-80
	5.6 Microbiological characterization	80-94
	5.6.1 Morphological characterization	80-84
	5.6.2 Biochemical characterization	85-94
	5.7 Antibiotic sensitivity profiling	94-99
	5.8 Genomic DNA isolation	100
	5.9 PCR amplification of 16S rRNA gene	101
	5.10PCR product purification by agarose gel extraction	102
	5.11Phylogenetic analyses	103-104
	5.12 Whole Genome analyses	105-106
	5.13 Virtual screening	106-114
7.	**6. Discussion**	115-120
8.	**7. Overall Summary**	121 - 122
9.	**Conclusion**	123

10.	**References**	124 - 141
11.	**APPENDIX:** **List of publications**	

ABSTRACT

Antimicrobial resistance (AMR) is a pressing global public health concern, ranking among the top 10 threats worldwide. The situation of AMR in India is particularly worrisome, with the country experiencing high levels of antibiotic consumption and resistance. Additionally, the COVID-19 pandemic has further exacerbated the issue, as the increased use of antibiotics may contribute to a more severe AMR crisis and may lead to the emergence of another pandemic.

The environment plays a vital role in the evolution and transmission of novel resistance genes in pathogens. Of particular interest are the environmental effluents or wastewaters which harbour ARGs and ARBs and therefore wastewater surveillance can provide valuable insights into the environmental reservoirs of resistance and aid in the development of effective strategies to mitigate AMR by identification and analyses of microbial abundance and potential pathogenic strains.

It is also vital to explore other strategies to combat AMR resistance. This can be done by studying plant-based secondary metabolites and identifying their antimicrobial potential. By screening crude plant extracts, we can identify bioactive compounds that interact synergistically with antibiotics, enabling their use in combination therapy and aid in future drug discovery prospects.

The objective of this research is to investigate the antimicrobial resistance profile in wastewater from the environment and utilize metagenomic approaches to map the microbial composition and functional characteristics and identify plant-based small molecules which can act as ligands against target proteins from MDR bacteria.

The study involved collection of environmental effluents from rural (LS) and urban (SS) regions of West Bengal, India viz. Purulia and Kolkata respectively, which were subjected to the following analyses: Metagenomics profiling, Physicochemical analysis, Microbiological characterization, Antibiotic sensitivity profiling, Molecular identification of MDR isolates. The study led to the isolation and identification of numerous XDR and MDR isolates that are prevalent in the sampling sites as well as are part of the urban and rural resistant reservoir of the state.

A predictive pipeline for estimating the Pathogenic Load using taxon set enrichment analysis was formulated which offers a cost-effective and time-efficient alternative to

the complete process of disease prevalence prediction by identifying taxon sets associated with pathogenicity.

Computational analyses and molecular docking studies were employed to identify new natural ligands with the potential to target disease modifier proteins. These findings hold great promise for future drug discovery efforts and offer hope in the fight against antibiotic resistance.

Keywords: AMR surveillance, antibiotic resistance, MDR, ARB, Metagenomics, phytocompounds, molecular docking

I. INTRODUCTION

1. Introduction

1.1 Emergence of antibiotic resistance

Antibiotics are the products of secondary metabolism of microorganisms such as bacteria and fungi.

One of the most important discoveries in the field of medical science is the discovery of penicillin by Alexander Fleming in the year 1929. The introduction of penicillin lead to the isolation of novel antibiotics rapidly during 1940s-1970s and over 160 new antibiotics and semi-synthetic derivatives molecules were commercialized. As a result, there was an accelerated decline in the number of infection related mortalities and morbidities (Shales and Bradford, 2018).

The miracle, however, is over today due to increasing reports of bacteria which can tolerate or resist the action of antibiotics.

Antibiotic resistance has become one of the most pressing public concerns in the recent times. In his Nobel Prize speech in 1945, Alexander Fleming expressed concern about antibiotic resistance, emphasizing its significance. Fleming, credited with the discovery of the first antibiotic, penicillin, highlighted the need to be cautious about the emergence of antibiotic resistance, "*It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body*" (Fleming, 1945).

According to an evaluation published in The Lancet by Murray (2022), the resistance to antibiotics poses a significant threat worldwide, resulting in a mortality rate that is expected to surpass that of HIV or malaria. The analysis reveals compelling data: in the year 2019, approximately 4.95 million individuals lost their lives due to bacterial infections that were resistant to drugs. Out of these fatalities, around 1.27 million can be directly attributed to antimicrobial resistance (AMR).

According to the report commissioned by the UK Government, known as the Review on Antimicrobial Resistance, it was argued that by the year 2050, antimicrobial resistance (AMR) could result in the deaths of approximately 10 million individuals annually (de Kraker et al., 2016).

The significance of enhancing global health readiness for potential threats, such as the COVID-19 pandemic, has underscored the importance of addressing the "silent pandemic" of antimicrobial resistance (AMR). AMR is already exerting substantial effects on both economies and healthcare systems, resulting in an estimated annual death toll of 700,000 worldwide due to infections resistant to drugs.

According to a study published in The Lancet (2022), it was discovered that in the year 2019, the prevention of all drug-resistant infections could have potentially saved 4.95 million lives. Alternatively, replacing drug-resistant infections with infections susceptible to drugs could have prevented approximately 1.27 million deaths.

Escherichia coli (*E. coli*) a exhibited significant amount of resistance towards cephalosporins and fluoroquinolones (third generation) which were responsible for the most deaths. *Klebsiella pneumoniae* showed a similar trend in terms of resistance. Interestingly, "Methicillin-resistant *Staphylococcus aureus* (MRSA)" was responsible for numerous deaths across the globe.

"Extended-spectrum beta-lactamases (ESBLs) found in Enterobacteriaceae have exacerbated the global challenge of antibiotic resistance. These enzymes hydrolyze and render beta-lactam antibiotics, such as third-generation cephalosporins, ineffective. Additionally, ESBL-producing bacteria have demonstrated simultaneous resistance to quinolones, aminoglycosides, and sulphonamides, further fueling the rise of multidrug resistance. For instance, within a hospital in Kenya, it has been observed that 90% of *E. coli* isolates producing ESBL have exhibited resistance to fluoroquinolones as well (Kawamura *et al.*, 2017)."

In South Asia, India experiences one of the highest age-standardized mortality rates for infectious diseases, and the levels of antibiotic resistance are concerning (Manesh and Varghese, 2021). In hospital-acquired infections, over 50% of *Klebsiella* spp isolates exhibit resistance to carbapenems. A notable portion of these strains also display resistance to polymyxins, resulting in a case fatality rate of nearly 70.4%.

At the same time, India holds the global lead in terms of human antibiotic consumption, which is a significant factor contributing to the development of antimicrobial resistance, with an average of 10.7 units per person (Taneja and Sharma, 2019). The prevalence of resistance leads to an escalation in the utilization of broad-spectrum empirical antibiotic treatment, resulting in limited options for effective treatment and deteriorating patient

outcomes. India's antimicrobial resistance issue is further exacerbated by factors such as unrestricted access to antibiotics, lack of knowledge, insufficient utilization of diagnostics, overcrowding, cross-infections, financial incentives for doctors from pharmaceutical companies, and inadequate healthcare infrastructure.

1.2 Role of wastewater in dissemination of antibiotic resistance in environment

While the use of antibiotics and the proliferation of antibiotic resistance in clinical environments are widely acknowledged concerns, the recognition of antibiotics and antibiotic resistance as environmental issues and pollutants has received less attention. This is likely because antibiotics in non-clinical settings are typically present at concentrations considerably lower than therapeutic usage levels. However, even low levels can sustain and/or favour development and spread of antibiotic resistance in microbial communities. Over the past decade, there has been a growing number of documented instances where antibiotics and antibiotic resistance genes have been detected in various environmental settings. Studies have indicated that the transfer of antibiotic resistance genes can transpire between bacterial strains that are evolutionarily and ecologically unrelated, even in the absence of antibiotics (Peterson and Kaur, 2018).

Antibiotic resistance genes exhibit wide distribution and diversity among environmental bacteria. However, in order to present a clinical risk to humans, these genes must enter and be expressed within strains belonging to the commensal flora of mammals or those capable of colonizing the mammalian gut, nasopharynx, lungs, or urinary tract, potentially leading to infections. One suggested scenario is that the anthropogenic utilization and dispersal of antibiotics result in artificially elevated concentrations within human environments. This, in turn, potentially facilitates the gene transfer process from environmental bacteria to pathogenic strains that affect mammals. Numerous studies have been conducted to examine the prevalence of resistance in commensal bacteria found in wild animals. These studies consistently indicate a correlation between resistance and the extent of exposure to humans or human-associated activities (van den Honert *et al.*, 2020).

“The antibiotics are dispersed in two ways, (1) urine and faeces, or (2) direct disposal. A significant portion of the antibiotics consumed is not absorbed or processed by the

body but instead eliminated in their active form through urine and feces. The urine and faeces are transported to wastewater treatment plants or can be used directly as manure. Direct disposal includes addition of food additives directly to the water in fish farms or treatment of crops. One large source is probably the disposal of outdated or remainders of antibiotics in household and farm drains. Wastewater treatment plants (WWTPs) are likely the main pathways through which antibiotics enter the environment. Multiple studies have documented the presence of various antibiotics in both untreated and treated water sources. Most of these studies indicate lower concentrations of antibiotics in treated water, indicating that there is partial removal of antibiotics in WWTPs. However, it has been indicated that biodegradation does not occur for all antibiotics in the WWTPs (Vasilachi *et al.*, 2021)."

The cumulative evidence regarding the selection of resistance in environments characterized by extensive antibiotic pollution (such as areas affected by antibiotic manufacturing) is significantly more robust than that for excreted antibiotics. This is supported by observations of selective concentrations surpassing normal levels by substantial margins at industrially polluted sites, a higher relative abundance of resistant bacteria, and notable increases in the number of antibiotic resistance genes (ARGs), including previously unidentified ones. Importantly, these increases in ARGs are not accompanied by corresponding increases in fecal contamination.

"In numerous instances, metals and antibacterial biocides can concurrently contribute to the selection of antibiotic-resistant strains through cross-resistance (via the same mechanism) or co-resistance (via genetically linked mechanisms) (Larsson *et al.*, 2022). The available evidence indicates that the current coexistence of metal and biocide resistance genes (MRGs and BRGs) and antibiotic resistance genes (ARGs) on plasmids is primarily attributed to the historical exposure to antibiotics, rather than exposure to metals or biocides." The significant co-occurrence of these resistance genes is predominantly observed in communities, such as the human and domestic animal microbiota, that have undergone extensive antibiotic selection pressures. The required concentrations for selection or co-selection of metals and biocides are not well-explored and require additional research. Similar to certain antibiotics and pharmaceuticals, certain biocides have been found to increase the rate of horizontal gene transfer (HGT).

"The presence of faecal bacteria in environmental contamination facilitates physical contact, creating more possibilities for gene exchange between environmental bacteria and those adapted to the intestinal tracts of humans or domestic animals. Furthermore, various intestinal bacteria serve as carriers of genetic elements such as integrative conjugative elements, plasmids, insertion sequences, integrons, or transposons, which play a role in facilitating the acquisition and transfer of genes to pathogens."

1.2.1 Antibiotic-resistant bacteria and genes in Sewage and Hospital wastewater

"Hospital wastewater contains substantial quantities of antibiotic-resistant organisms. This is primarily attributed to the release of antibiotics through the excretion of used medications and improper disposal of unused compounds, which can subsequently find their way into the environment. The presence of antibiotics in aquatic ecosystems can contribute to the proliferation of antibiotic-resistant bacteria. Hospital effluents, including wastewater treatment plants associated with hospitals, serve as significant sources for the emergence of both antibiotics and antibiotic-resistant bacteria into the environment."

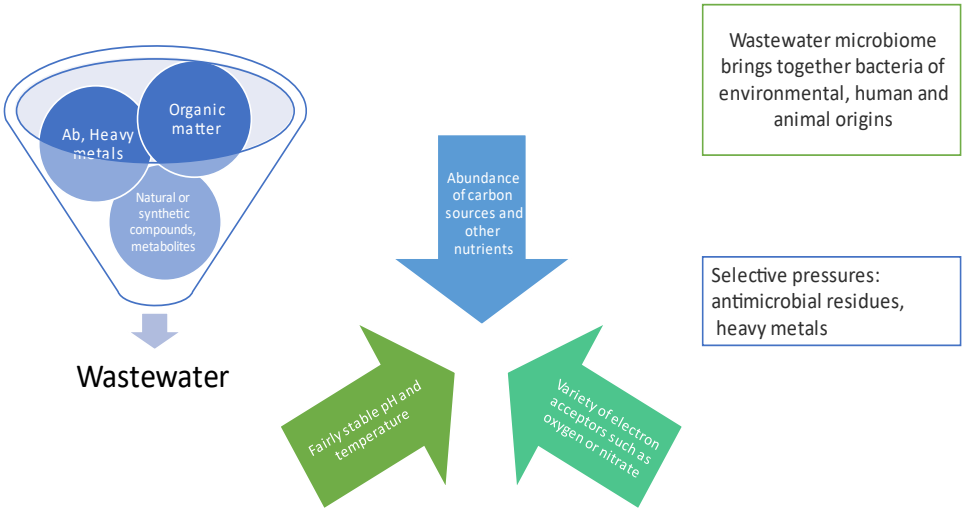


Fig. 1.1: Wastewater microbiome: ideal environment for proliferation and transmission of ARB and ARGs

Lamba et al., 2017 discovered that ARB and ARGs present in hospital wastewaters in New Delhi pose a specific health hazard due to the presence of highly multi-drug resistant (MDR) phenotypes, including pathogens that are classified as "untreatable."

Another study conducted in 2019 aimed at the investigation of microbiota and antibiotic resistome of hospital effluent collected from the city of Mumbai, India. Through the utilization of hidden Markov models on shotgun data and amplicon sequencing of integron gene cassettes, numerous new antibiotic resistance genes (ARGs) were identified. The investigation revealed the presence of various functional carbapenemases in E. coli, which were identified through their expression in the bacterium (Marathe et al., 2019).

"Praveen kumar Reddy *et al.* in 2020 investigated the distribution of antimicrobial-resistant Escherichia coli (E. coli) in four sewage treatment plants (STPs) in South India receiving hospital and domestic wastewater in different proportions. The study revealed that there was no significant disparity observed between the wastewater inlet and outlet, indicating that the treatment process employed was ineffective in reducing resistance. Furthermore, the research indicated that the levels of resistance were slightly elevated in hospital wastewater compared to domestic wastewater."

In a thorough investigation by Talat et al. in 2023 using "shotgun metagenomics, the antibiotic resistomes present in wastewater samples from six different hospitals in various rural and urban regions of northern India were comprehensively examined. The study highlighted the prevalence of antibiotic resistance genes (ARGs) targeting aminoglycoside, macrolide, carbapenem, trimethoprim, and sulfonamide antibiotics in all analyzed samples, as confirmed by both read-based and assembly-based analyses."

1.2.2 Antibiotic-resistant bacteria and genes in Rivers

"In India, significant levels of antibiotic resistance have been observed in major rivers, particularly against broad-spectrum antibiotics like third-generation cephalosporins. In a study conducted on the River Kaveri in Karnataka, it was found that all E. coli isolates were showing resistance to third-generation cephalosporins (Skariyachan et al., 2015). Another research focusing on the River Yamuna found that 17.4% of isolates which belong to various groups of gram-negative bacteria were ESBL producers (Azam et al., 2016). Moreover, the presence of antibiotic resistance genes (ARGs) associated with broad-spectrum antibiotics, including last-resort agents, was found in these rivers. (Azam et al., 2016; Akiba et al., 2016; Devarajan et al., 2016; Ahmad et al., 2014; Marathe et al., 2017). These findings narrate the significance of antibiotic-resistant bacteria and ARGs in India's major rivers."

"In 2021, a team of scientists from Allahabad University Centre of Environmental Sciences found ampicillin-resistant isolates (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Aeromonas hydrophila*, and *Acinetobacter baumannii*) while two isolates as erythromycin-resistant (*Streptococcus pneumoniae* and *Enterococcus faecalis*) in municipal sewage water joining river Ganga, at Prayagraj (India). (Kumar *et al.*, 2021)."

A study conducted on E. coli strains from the Yamuna River demonstrated a significant occurrence of drug resistance. In this study, "the presence of the class 1 integron gene intI was detected in 75% of the isolates (Singh et al., 2021)."

1.2.3 Antibiotic-resistant bacteria and genes in Surface water and Groundwater

Research has demonstrated that potable water sources, in addition to rivers, can harbor bacteria exhibiting elevated levels of resistance to broad-spectrum antibiotics.

When it comes to the acquisition of new resistance elements, water, soil, and other environments characterized by diverse ecological niches offer an unparalleled gene pool with a richness and variety that surpasses that found within the microbiota of humans and domestic animals.

Undoubtedly, the remarkable characteristic of the environmental microbiome lies in its vast diversity, offering a multitude of genes that have the potential to be acquired and employed by pathogens as a defence mechanism against the effects of antibiotics.

To date, "resistance has emerged in pathogens targeted by all approved antibiotic classes, regardless of whether they are natural, semi-synthetic, or synthetic compounds. This indicates that external environments already contain resistance elements for all antibiotics that will be developed in the future unless we adopt a radically different approach to antibiotic design."

1.3 Environmental Surveillance of antibiotic resistance

It is crucial to conduct systematic monitoring of antibiotic usage and the prevalence of antibiotic resistance in both humans and animals to effectively manage bacterial infectious diseases. To combat the growing risk of drug-resistant infections and diseases, the field needs to transition towards collaborative efforts in comprehending

the environmental presence, human exposure, efficient mitigation strategies, and ultimately conducting dose-response assessments for human health risk evaluations.

To accomplish these objectives, it is essential to create universally applicable standardized methods that can be utilized at both local and global levels. These methods would facilitate the establishment of baseline data on the environmental presence of relevant water environments, serving as a critical initial step towards achieving the aforementioned goals.

1.3.1 Culture-Based Methods

According to Acharya et al. (2021), it is estimated that normal soil contains approximately 10^9 to 10^10 bacteria per gram of dry weight. However, it is important to note that only a small percentage, around 1%, of these bacteria can be isolated and purified using culture-dependent techniques. This highlights the vast diversity and complexity of bacteria present in soil ecosystems, with the majority of bacterial species remaining unculturable using traditional laboratory methods.

1.3.1.1 Importance of culture media for growth and purification of bacteria

In microbiology, a pure culture refers to a laboratory culture that contains only a single species of organisms. The process of preparing a pure bacterial culture involves selecting the appropriate culture media based on the characteristics of the bacteria. Having a purified culture is essential for conducting morphological and phylogenetic studies of bacteria. A proper culture media should provide all the necessary environmental components required for the growth and multiplication of a specific bacterium, mimicking its natural habitat as closely as possible. This allows researchers to study the characteristics, behaviour, and interactions of individual bacterial species in a controlled laboratory environment.

Culture media play a vital role in various microbiological tests and experiments. They serve several purposes, including obtaining pure cultures, growing, and counting microbial cells, and cultivating and selecting specific microorganisms under controlled laboratory conditions. The term "culture" refers to the intentional growth of a particular microorganism in a laboratory setting, away from its natural environment.

For accurate and reliable microbiological test results, high-quality culture media are essential. A microbiological culture medium is a substance designed to support the

growth, survival, and replication of microorganisms. It contains a combination of nutrients, growth factors, energy sources, buffer salts, minerals, metals, and gelling agents (in the case of solid media). These components provide the necessary conditions and resources for microorganisms to thrive and carry out their metabolic activities.

Microbial cultures serve as foundational tools in microbiology and molecular biology research. They enable scientists to isolate and study specific microorganisms, investigate their characteristics, conduct diagnostic tests, and perform genetic and biochemical analyses. By providing an environment that supports the growth of desired microorganisms while inhibiting the growth of unwanted contaminants, culture media facilitate the study and manipulation of microorganisms in the laboratory.

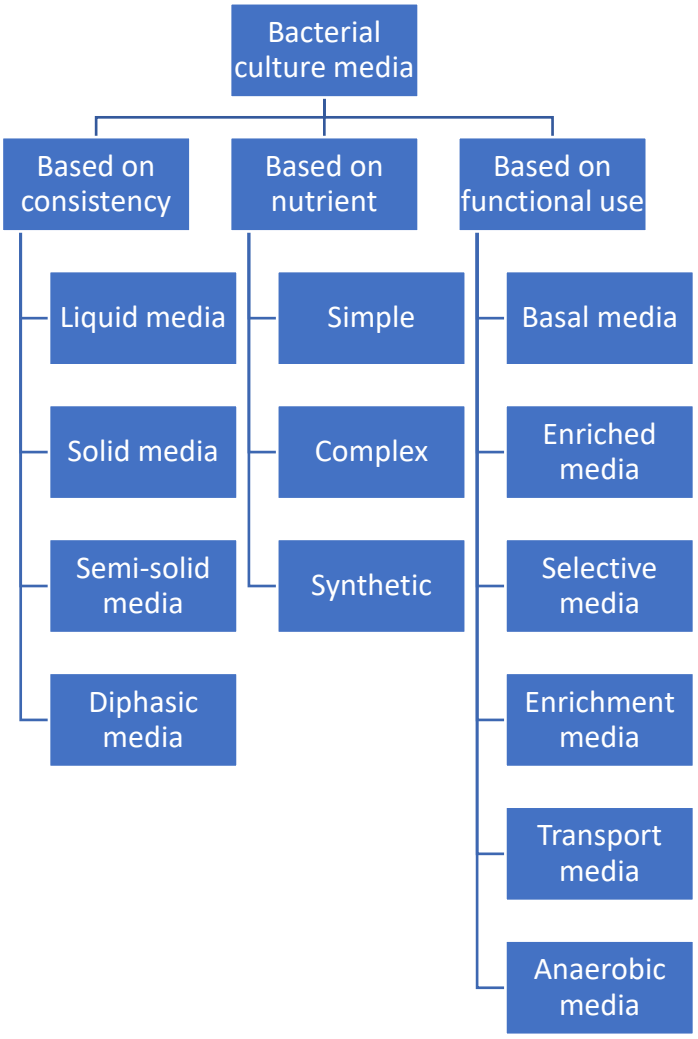


Fig. 1.2: Types of Culture media used in the microbiological characterization of microorganisms

1.3.1.2 Characterization of bacteria

Identification and characterization of bacteria rely on studying their cellular morphology, as well as their physiological and biochemical properties. The classification of bacteria based on these features is a practical approach to identifying and differentiating these organisms. Each microbe possesses distinct physiological and biochemical characteristics, which are determined by the presence and expression of specific genes. These genes control the activity of enzymes involved in the biosynthesis and biodegradation of compounds.

By examining the morphological features of bacteria, such as their shape, size, and arrangement, we can gain initial insights into their classification. However, to obtain a more accurate identification, it is necessary to analyse their physiological and biochemical properties. This involves studying various aspects of their metabolism, such as their energy sources, nutrient requirements, growth conditions, and their ability to carry out specific biochemical reactions.

Each microbe possesses its unique combination of biochemical and physiological traits, which serves as its "fingerprint" and distinguishes it from other species. This individuality allows for the identification and differentiation of bacteria based on their specific features, aiding in their classification, and understanding of their roles in various biological processes.

1.3.1.3 Morphological Study of Bacteria

In the preliminary characterization of bacteria, the first step involves classifying them based on their cellular and colony morphology. Cellular morphology refers to the physical appearance of bacterial cells, which can be observed under a microscope after appropriate staining techniques. Bacterial cells can occur either as individual cells or in aggregated forms. The most common shapes observed in bacterial cells are spherical (Coccus) or rod-shaped (Bacillus). However, certain bacteria exhibit unique cellular morphologies, such as comma-shaped (Vibrio) or spiral-shaped (Spirillum). When bacterial cells multiply, they form visible colonies. These colonies can be observed on solid culture media after incubation. Estimating the viable cell count in a bacterial population can be achieved by counting the number of colonies that develop. This estimation is expressed as colony-forming units (CFUs), which represent the approximate number of viable cells. The colony morphology, including its size, shape, colour, texture, and other visible characteristics, can vary among different bacterial species. This morphology is influenced by the association and arrangement of colony

members, as well as their physical and biochemical properties. By analyzing colony morphology, microbiologists can gain initial insights into the characteristics of a bacterial species. However, it is important to note that colony morphology alone is not sufficient for definitive identification and classification of bacteria. Further tests and analyses, including physiological and biochemical assays, are required for more accurate characterization and identification of bacterial species.

1.3.1.4 Role of Culture-based Methods in AMR surveillance

"Culture-based methods are appealing for monitoring AMR since they allow for the selection of specific targets with clinically recognized significance (e.g., taxonomic groups containing human pathogens), methods are well standardized for defining clinical resistance levels (e.g., EUCAST, CLSI, Kirby-Bauer), and, by definition, the recovered target is viable (Ligouri *et al.*, 2022)."

Culture-based assays are particularly well-suited for providing valuable information to inform human health risk assessments.

A difficulty encountered with culture-based methods for monitoring antimicrobial resistance (AMR) is that while there are various genera/species found in wastewater and surface water environments that could provide valuable information, the high levels of background microorganisms in these environments can potentially disrupt the isolation methods used for the desired target.

Furthermore, the majority of environmental microbes cannot be easily cultured, which means that culture-based methods may overlook the broader spectrum of resistance present within each environmental reservoir. It is important to note that no single target can comprehensively capture the antimicrobial resistance (AMR) status of a specific environment.

1.3.2 Community Genomics-Based Methods

Classification of living organisms initially started with analysing the similarities and differences in their phenotypic characters. With the advancement of molecular biology-based techniques and computational science, accurate inference on the phylogenetic classification of living organisms became gradually dependent on the molecular characteristics of the specimens.

1.3.2.1 Importance of 16S rRNA gene

The 16S rRNA gene, encoding a component of the ribosome has been found to possess several characteristics that make it an ideal target for phylogenetic analysis. Here are the reasons behind its widespread use:

- Ubiquitous Presence: The 16S rRNA gene is present in all bacteria, often as multiple copies within the genome. This gene's presence allows for its universal application in studying the evolutionary relationships among bacteria.
- Conserved Function: The function of the 16S rRNA gene, which is involved in protein synthesis, is highly conserved across bacteria. Its crucial role in cellular processes suggests that changes in the gene sequence can provide insights into evolutionary relationships.
- Sequence Variation: Although the overall function is conserved, the 16S rRNA gene exhibits sufficient sequence variation between bacterial species, allowing for the differentiation and classification of different taxa. Comparing the differences in the gene sequence enables the estimation of evolutionary distances and the construction of phylogenetic trees.
- Length of the Gene: The 16S rRNA gene is approximately 1500 base pairs in length, which provides a sufficient amount of sequence information for accurate phylogenetic analysis. This length allows for meaningful comparisons between different species and facilitates the use of computational tools and algorithms for sequence alignment and phylogenetic inference.

Due to these factors, the 16S rRNA gene has become the most commonly used housekeeping gene for phylogenetic analysis of bacteria. Its wide distribution, functional conservation, sequence variability, and appropriate length make it an invaluable tool for understanding bacterial evolution and taxonomy.

This housekeeping gene consists of patches of unique sequences or signature sequences with some universal sequences in between. These universal sequences are selected for designing the universal 16s-specific primers. Though there are many different primers for the amplification of 16S rDNA fragments, the most used six universal 16S specific primers are given in Table.

Primer position	**Sequence**
27 forward	5’AGAGTTTGATCCTGGCTCAG 3’
341 forward	5’ACTCCTACGGGAGGCAGCAG 3’

515 forward	5’ TGCCAGCAGCCGCGGTAA 3’
907 reverse	5’AAACTCAAAGGAATTGACGG 3’
1190 reverse	5’AGGAAGGTGGGGATGACGTC 3’
1492 reverse	5’ TACGGTTACCTTGTTACGACTT 3’

Table 1.1: Sequence of six universally used 16s gene-specific primers

1.3.2.2 Databases used

There are several quality-controlled databases available for the 16S rRNA gene sequences of bacteria. Here are some commonly used databases:

- **GenBank**: GenBank, hosted by the National Center for Biotechnology Information (NCBI), is one of the most comprehensive and widely accepted databases for biological sequences, including 16S rRNA gene sequences. It provides a vast collection of publicly available sequences and associated metadata. GenBank is continuously updated with new submissions from researchers worldwide, making it a valuable resource for comparing and annotating sequencing results.
- **Ez Taxon**: Ez Taxon is a web-based database that focuses specifically on bacterial 16S rRNA gene sequences. It provides a user-friendly platform for taxonomic identification and classification of bacterial isolates based on their 16S rRNA gene sequences. Ez Taxon incorporates high-quality, curated reference sequences to ensure accurate and reliable identification.
- **BIBI**: The BIBI (Base d'Identification des Bactéries du lnnstitut Pasteur) database is maintained by the Pasteur Institute and provides a curated collection of bacterial 16S rRNA gene sequences. It offers a comprehensive set of aligned and annotated sequences, along with various tools for phylogenetic analysis and identification of bacterial isolates.

While GenBank is a widely accepted and frequently used database, it is generally recommended to analyze 16S rRNA gene sequencing results using multiple databases. This helps to ensure robustness and accuracy in taxonomic assignments and comparisons. GenBank's comprehensive nature makes it a valuable resource, but incorporating additional databases like Ez Taxon and BIBI can provide complementary information and help validate the results.

1.3.2.3 Role of Molecular-based Methods in AMR Surveillance

Molecular-based assays that focus on detecting ARGs offer certain advantages over phenotypic assays. These advantages include the ability to target multiple genes simultaneously (multiplex targeting) and providing more accurate characterization and detection of specific AMR genes.

Molecular-based methods offer a suitable alternative in taxonomic units where susceptibility breakpoints have not been determined. Another benefit is the elimination of the need for isolate purification, as molecular-based methods allow for the use of non-purified polymicrobial samples. Furthermore, they enable prompt adjustment to newly emerged resistance factors. Molecular-based techniques provide a rapid and highly sensitive means of detecting ARGs. (Kaprou *et al.*, 2021).

1.3.3 Metagenomics-based methods

The advent of next-generation DNA sequencing (NGS) applications in 2009 revolutionized the field by enabling the profiling of ARGs and other genes present in an environmental sample without the need for pre-selecting specific targets. This technique is known as metagenomic sequencing.

Metagenomic sequencing involves the direct extraction of genomic DNA from environmental samples, followed by fragmentation and the application of NGS technology. This process generates millions of reads that represent the bacterial community present in the samples.

Initially, metagenomics was employed to study river sediments in surface waters that were heavily influenced by pharmaceutical wastewater discharges, with a specific focus on antibiotic contamination. In a recent study conducted by Liguori et al. (2022), researchers discovered elevated levels of antimicrobial resistance genes (sul2, aph-, tet-, qnr-, erm-), transposons, plasmids, and integrons in water samples. These factors pose a significant risk for horizontal gene transfer (HGT) and contribute to the spread of antimicrobial resistance.

“Metagenomics is now widely applied for examining shifts in the resistome (i.e., the collection of all ARGs across an environmental sample) through WWTPs and identifying MGEs in order to estimate the extent of HGT events. Metagenomics has also been used to characterize resistomes and treatment efficiencies in recycled water

and drinking water treatment plants." In recent times, the potential of metagenomics as a powerful tool for wastewater-based epidemiology has been acknowledged. This approach involves monitoring raw sewage to estimate the presence of ARGs and the shedding of these genes by corresponding human populations. (Ligouri, 2022).

1.4 Plant-derived antimicrobials for therapeutic purposes

The rise in bacterial resistance to antibiotics has led to a decline in the discovery of new antimicrobial agents. As a result, researchers have turned their attention towards alternative therapies, such as traditional plant-based medicines, bacteriophage therapies, and combination therapies, in search of potential solutions. The rise of antibiotic resistance in disease-causing bacteria has sparked a renewed interest in investigating the potential of plant-derived antimicrobials (PDAs) as an alternative approach to address microbial infections.

Traditional medicine has a long history of utilizing plant extracts as a natural and safe solution for treating various ailments and diseases. Out of the various available options, plant-derived compounds have shown considerable promise in the fight against bacterial infections. Plant-derived chemicals encompass a diverse range of naturally occurring compounds found in plants. These compounds have demonstrated significant advantages, including antioxidant, antibacterial, and antifungal properties. (Khameneh et al., 2019).

The main benefit of utilizing PDAs for therapeutic purposes is their reduced likelihood of causing side effects commonly associated with synthetic chemicals. Furthermore, as far as current knowledge suggests, there have been no reported instances of antimicrobial resistance against these phytochemicals. This is likely due to their multiple mechanisms of action, which help prevent the development of resistant bacterial strains (Vaou *et al.*, 2021). The notable antimicrobial properties, non-toxic characteristics, and affordability of these compounds have established their extensive utilization. Plant extracts offer extensive applications as growth promoters in the livestock and poultry industry, as effective antimicrobials and disinfectants in the food industry, as essential components of herbal therapy in veterinary medicine, and as valuable resources for the discovery and development of novel antibiotics in the pharmaceutical field.

In the current postgenomic era, the integration of diverse genomic disciplines offers the potential to uncover new targets for the development of inhibitors. Computational approaches and the utilization of "omics" data, including metabolomics, genomics, and proteomics, have significantly enhanced the prospects of identifying relevant targets for pharmacological purposes (Caudai et al., 2021).

Molecular docking is a promising approach in drug discovery and design, as it provides valuable insights into the interaction between drugs and proteins/DNA. By simulating the binding process and forming stable complexes with high efficacy and specificity, molecular docking serves as a valuable tool for identifying new drug targets. This approach makes molecular docking a highly efficient technique for identifying and synthesizing novel drugs with significant potential (Adelusi et al., 2022).

Currently, molecular docking is utilized to rationalize the activity of ligands towards a specific target of interest and to conduct structure-based virtual screening. It assists in understanding how ligands interact with the target and aids in the identification of potential lead compounds for further exploration. Docking techniques are also utilized to identify ligands that have the ability to bind to multiple selected targets simultaneously (polypharmacology) and to discover new applications for chemical compounds that already have well-established safety profiles (drug repositioning). This approach enables the exploration of potential interactions between ligands and multiple targets, as well as the repurposing of existing drugs for new therapeutic purposes.

Additionally, molecular docking can be employed to identify a series of targets that show good complementarity with specific ligands (target fishing and profiling). This approach can help identify potential off-target effects of drugs, which can be responsible for unexpected adverse reactions. By examining the interactions between ligands and various targets, docking techniques provide insights into the potential interactions and effects of drugs beyond their intended targets (Pinzi and Rastelli, 2019).

To initiate a docking calculation, the first requirement is to acquire the structure of the target, which typically involves a significant biological molecule such as a protein, DNA, or RNA. These macromolecular structures can be easily obtained from the Protein Data Bank (PDB), a comprehensive resource that offers access to three-dimensional atomic coordinates obtained through experimental techniques.

"The structure of the ligand is also necessary and can be obtained from databases that contain information on small molecules, such as ZINC and PubChem. These online databases provide easy access to a wide range of compounds that can be used for virtual screening. In cases where the three-dimensional atomic coordinates of the compounds are not directly available, they can be derived from their two-dimensional structures or simpler representation schemes like SMILES. This process starts with the discovery of molecules that show efficacy in a simple screen, called "hits." Screening is a procedure in which a large number of compounds, including those derived from natural products and available in online databases, are evaluated for their biological activity using high-throughput assays."

"Docking methods involve the fitting of a ligand into a binding site by optimizing various factors such as steric, hydrophobic, and electrostatic complementarity. These methods also estimate the free energy of binding, known as scoring, to assess the strength of the ligand-receptor interaction."

2. LITERATURE REVIEW

2. Literature Review

2.1 Antibiotic Resistance as an environmental problem

Paul Ehrlich introduced the concept of using antimicrobial compounds in 1908 with the idea of employing specific chemicals, which he referred to as "magic bullets," to target and eliminate bacteria while minimizing harm to the host (animals and humans). He developed a treatment called Salvarsan (also known as arsphenamine) for syphilis. Salvarsan was derived from arsenic compounds and was introduced in 1910 as an effective therapeutic option for syphilis. (Ehrlich, 1910). This marked the first instance of employing a chemical compound for the treatment of microbial infections.

Following the breakthrough of penicillin, scientists dedicated significant efforts to uncovering additional antibiotics, resulting in the identification of various new ones such as tetracycline, gentamicin, and chloramphenicol. As a result, until the 1950s, the majority of human infectious diseases could be effectively treated using these antibiotics (Hutchings *et al.*, 2019).

However, shortly after the introduction of antibiotics for clinical use, a concerning phenomenon emerged in *Staphylococcus aureus*. This bacterium developed a resistance mechanism that rendered it no longer susceptible to penicillin. It began producing an enzyme called penicillinase, which effectively dismantled the crucial beta-lactam ring of penicillin. This ring is essential for binding to bacteria through penicillin-binding proteins (PBPs) and exerting its antimicrobial effects. This development marked the emergence of antimicrobial/antibiotic resistance in bacteria.

Over the past century, extensive research efforts have yielded numerous new antibiotics. However, starting from the 1990s, there has been a significant decline in the discovery of new antimicrobial agents, coinciding with a troubling rise in the occurrence of antibiotic resistance. The prevalence of bacteria that are resistant to multiple classes of antibiotics, known as multidrug-resistant (MDR) bacteria, has become widespread, particularly in hospital settings. This alarming trend raises concerns about the potential emergence of a "post-antibiotic era" in the near future, where infections that were once easily treatable could become life-threatening. Antibiotic resistance is now widely recognized as a significant health issue, with substantial clinical and economic implications (Terreni *et al.*, 2021).

2.1.1 Bacteria level

Bacteria undergo constant evolutionary changes to develop resistance against antibiotics, which is a direct consequence of the ongoing battle between microbes. Bacteria are naturally inclined to develop mechanisms of resistance to counteract the effects of antimicrobial agents present in their environment, such as soil. This intrinsic resistance refers to the presence of resistance genes within the bacterial genome. These genes can be activated through human, agricultural, or animal use of antibiotics, or they can be acquired through mutations or a gene transfer from other bacterial species, a phenomenon known as acquired resistance (Reygaert, 2018).

2.1.2 Individual human level

The human commensal microbiome refers to the collection of non-harmful bacteria that reside in various body sites, such as the gastrointestinal tract (GIT) and skin. This microbiome is established in neonates from the very beginning, even on the first day of life. In the absence of factors that exert selective pressure, such as exposure to antibiotics, both bacteria that are resistant to antibiotics and those that are susceptible coexist peacefully within the microbiome (Thursby and Juge, 2017).

Following the administration of antibiotics, selective pressure is exerted on the bacteria in the microbiome. This pressure can lead to the emergence of antibiotic resistance in naturally susceptible bacteria. The resistance can arise through two main mechanisms: either by acquiring genes that encode resistance through mutation or through the transfer of genetic material from other bacteria that are already resistant. This transfer can occur through processes like conjugation, transduction, or transformation. As a result, the use of antibiotics by an individual increases the likelihood of resistance development and reduces the population of susceptible bacteria within the microbiome (Ramirez *et al.*, 2020).

2.1.3 Population level

A substantial quantity of antibiotics, amounting to millions of metric tonnes, has been produced and used for various purposes in human healthcare, veterinary medicine, and agriculture. This extensive usage has played a significant role in the selection and proliferation of antibiotic-resistant bacteria, thereby contributing to the problem of antibiotic resistance (Manyi-Loh *et al.*, 2018).

The relationship between antibiotic use and resistance is intricate and influenced by several factors that can confound the connection. These factors include:

- Bacterial factors: Different types of bacteria exhibit varying levels of susceptibility and ability to develop resistance. Factors such as mutation rates and interactions between pathogens and their hosts also play a role.
- Human factors: Human-to-human transmission of resistant bacteria, as well as the impact of vaccination on reducing the spread of resistant strains, contribute to the overall picture. Individual behaviours and practices related to antibiotic use also influence the development and spread of resistance.
- Public health factors: Antibiotic resistance can be influenced by practices in food-producing animals, where the use of antibiotics is common. International travel to regions with high levels of antibiotic resistance can also contribute to the dissemination of resistant strains. Additionally, the effectiveness of sanitation measures in preventing the spread of resistant bacteria is a relevant public health consideration.

Taken together, these factors interact in complex ways, making it important to consider a holistic approach when addressing the issue of antibiotic resistance. (Aggarwal et al., 2023).

2.1.4 The Environmental Resistome

The antibiotic resistome refers to the collective set of genes and genetic elements associated with antibiotic resistance within microbial communities. It includes various types of resistance genes, both acquired from external sources and inherent within the genome (intrinsic resistance genes). Additionally, the resistome encompasses precursor genes that may evolve or undergo changes in their expression context to confer resistance. These genes and mechanisms within the resistome contribute to the overall capacity of microbial communities to withstand the effects of antibiotics and survive their exposure (Kim and Cha, 2021).

The combined pool of bacterial antibiotic resistance genes is believed to have served as the source of resistance traits that have been acquired by pathogenic strains. This finding highlights that multidrug resistance in environmental bacteria is more widespread than previously thought. The presence of a diverse range of resistance

determinants in environmental bacteria suggests that these populations harbour a significant reservoir of resistance genes that can be transferred to pathogenic strains. This emphasizes the importance of understanding and monitoring the prevalence and distribution of antibiotic resistance in environmental bacteria for effective control and management of multidrug-resistant infections (Kunhikannan et al., 2021; Peterson, 2018).

"Increased dissemination of ARB and ARGs in the environment may result from selective pressures imposed by human activities. These activities include the overuse of antibiotics in clinics, hospitals, and nursing homes (Duan et al., 2020; Feng et al., 2017). Finally, antibiotics usage led to prevent diseases and promote growth in farm animals and aquaculture (Watts et al., 2017; Xiong et al., 2018)."

ARB and ARGs originating from these human activities accumulate in various locations such as wastewater treatment plants (WWTPs), wastewater, compost heaps, and water runoffs from farms. These sites serve as reservoirs where antibiotic-resistant bacteria and the genes responsible for antibiotic resistance gather and persist. These locations are particularly susceptible to receiving and harbouring ARB and ARGs due to their direct or indirect exposure to human activities involving the use of antibiotics. The presence of ARB and ARGs in these environments poses a potential risk for the spread of antibiotic resistance to other ecosystems and potentially to human populations. (Dong et al., 2019; Hendriksen et al., 2019; Karkman et al., 2018; Wu et al., 2019).

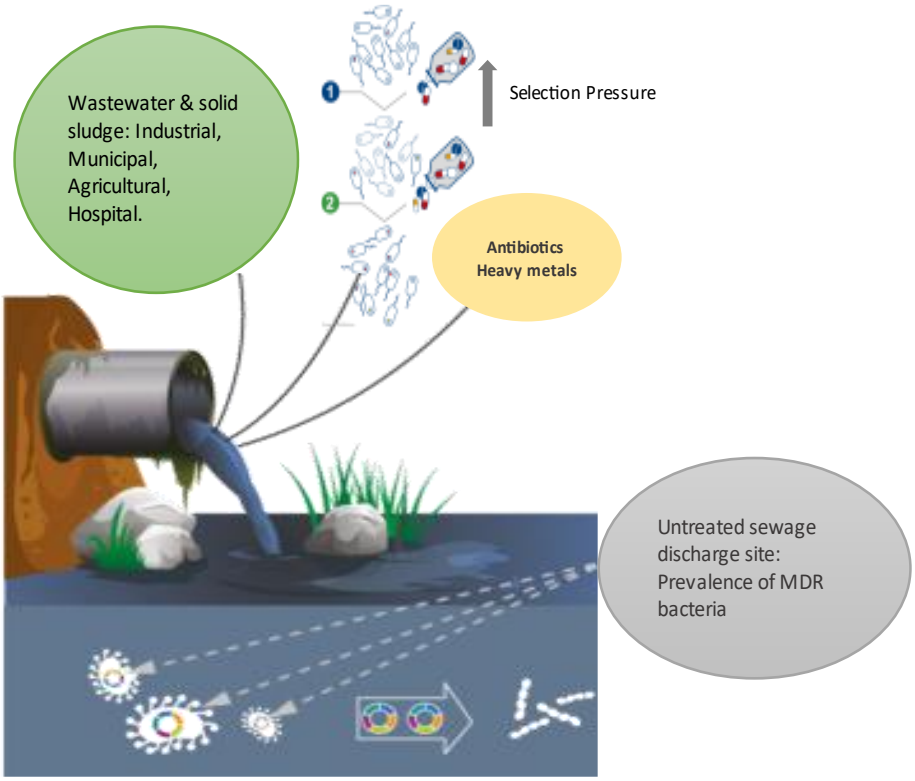


Fig. 2.1: Environmental wastewater & associated sludge: Biomarkers for global AR surveillance and prediction (Hendriksen, 2019)

"As the ARB die, the ARGs degrade or dissipate, but others may accumulate in water (Anthony et al., 2020; Bai et al., 2019; Yang et al., 2017), soil (Chen et al., 2016; Han et al., 2016; Wu et al., 2020), or air (Brągoszewska and Biedroń, 2018; Pal et al., 2016; Zhang et al., 2019). Different habitats may accumulate specific ARGs (Zhuang, 2021)."

In terms of microbial ecology, "the environment can be categorized into two main types: natural environments and built environments. Natural environments can further be classified into aquatic and terrestrial environments. The aquatic environment encompasses marine ecosystems (such as oceans and estuaries) as well as freshwater ecosystems (including rivers, lakes, and wetlands). On the other hand, the terrestrial environment encompasses diverse terrestrial ecosystems found in different climate zones, such as forests, deserts, grasslands, and tundra."

Built environments, on the other hand, comprise human-made settings and structures. These include wastewater treatment plants (WWTPs), agricultural sites, aquaculture operations, and hospital environments. These constructed environments provide

specific habitats and conditions that can significantly impact microbial communities and the dissemination of antimicrobial resistance.

Overall, understanding the dynamics of antimicrobial resistance in both natural and built environments is crucial for comprehending the broader ecological context and developing effective strategies for mitigating the spread of resistance. (Kim and Cha, 2021).

- **Soil resistome**: The soil environment, in its natural state, is considered a significant repository of the antibiotic resistome, encompassing both intrinsic resistance genes that naturally exist within bacteria and acquired resistance genes that are acquired through various mechanisms (Delgado-Baquerizo, 2022).
- **River resistome**: "Freshwater environments, including rivers, are considered reservoirs and dissemination routes for AMR. Several studies have clearly depicted a larger increase in ARGs in human-impacted river sites than in the pristine river sites, indicating the effects of anthropogenic activities on the river resistome. The resistome found in rivers holds significance due to its potential to reintroduce antimicrobial resistance (AMR) in human populations. As rivers serve as major sources of drinking water and irrigation water for agriculture, the presence of AMR in these environments poses risks. Additionally, the presence of novel contexts of antibiotic resistance genes (ARGs) in riverine ecosystems indicates the evolution and adaptation of ARGs for transmission, leading to significant challenges when they are reintroduced into human populations (Wang, 2022)."
- **Wastewater and WWTP**: Wastewater and wastewater treatment plants (WWTPs) are recognized as hotspots for the proliferation of ARB and ARGs. These environments provide favourable conditions for the survival and dissemination of ARB and ARGs, due to the high concentration of antibiotics and other selective pressures such as the presence of antibiotics, metals, and disinfectants present in wastewater. The presence of diverse microbial communities, along with the potential for horizontal gene transfer, contributes to the amplification and spread of ARB and ARGs in these settings. Efforts to monitor and mitigate ARB and ARGs in wastewater and WWTPs are crucial to minimizing the dissemination of antibiotic resistance. The significance of

wastewater as a reservoir of antibiotic resistance genes (ARGs) is increasingly recognized, particularly due to the growing number of reports on the discovery of novel ARGs, some of which possess mobility elements. Additionally, the emergence of antibiotic-resistant pathogens and ARGs in clinical settings has raised concerns about the potential role of wastewater in the transmission of antibiotic resistance. The presence of diverse microbial communities, the exposure to various antibiotics, and the potential for horizontal gene transfer in wastewater contribute to the dissemination and persistence of ARGs. Understanding the dynamics of ARGs in wastewater is crucial for effective management and control strategies to prevent the spread of antibiotic resistance. (Osińska *et al.*, 2021).

- **Animal resistome**: Extensive research has been conducted on the resistomes of livestock animals, and regional studies have demonstrated a link between antibiotic usage and the profiles of resistomes in these animals. Furthermore, cross-sectional studies examining the resistomes of farm and slaughterhouse workers who are exposed to animals have indicated a potential influence of the animal resistome on the human resistome. These findings highlight the significance of surveillance programs aimed at monitoring AMR in livestock animals. Understanding the transmission dynamics of AMR between animals and humans is crucial for implementing effective strategies to mitigate the spread of antibiotic resistance and ensure the safety of both animal and human populations (Gompel *et al.*, 2019).
- **Human resistome**: "Metagenomics, utilizing next-generation sequencing technology, has been employed to analyse the resistomes of human microbiomes, including those of the gut, skin, and respiratory tract. The resistome refers to the collection of ARGs present within the commensal bacterial community of the human microbiome. It is recognized as a significant reservoir and potential route for the dissemination of clinical ARGs. By studying the resistomes of human microbiomes, researchers aim to better understand the prevalence, diversity, and transmission of antibiotic resistance in the context of human health. This knowledge can contribute to the development of strategies for monitoring and managing antibiotic resistance in clinical settings (Brinkac *et al.*, 2017)."

2.2 Classes of Antibiotics and subsequent resistance development in Pathogens

Antimicrobial resistance mechanisms can be broadly categorized into four main types: (1) mechanisms that restrict the entry of drugs into bacterial cells, (2) mechanisms that alter or modify the target sites of drugs, (3) mechanisms that inactivate or destroy drugs, and (4) mechanisms that actively pump out drugs from the bacterial cells, known as drug efflux. These categories encompass the various ways in which bacteria can develop resistance to antimicrobial agents, allowing them to survive and continue to proliferate even in the presence of these drugs.

Intrinsic resistance can involve mechanisms such as limiting the uptake of drugs, inactivating drugs, and drug efflux from bacterial cells. On the other hand, acquired resistance mechanisms may include modifying the drug targets, inactivating drugs, and utilizing drug efflux. Intrinsic resistance refers to the inherent resistance of bacteria due to their natural characteristics, while acquired resistance refers to resistance acquired through genetic changes or the acquisition of resistance genes from other bacteria (Reygaert, 2018).

Gram-negative bacteria and gram-positive bacteria exhibit differences in their antimicrobial resistance mechanisms due to variations in their cell structure and composition. Gram-negative bacteria employ all four main resistance mechanisms, including limiting drug uptake, modifying drug targets, inactivating drugs, and active drug efflux. In contrast, gram-positive bacteria, which lack an outer membrane composed of lipopolysaccharides (LPS), less commonly utilize the mechanism of limiting drug uptake. Additionally, some types of drug efflux mechanisms may not be present or as prevalent in gram-positive bacteria due to differences in their cell membrane structure. These differences contribute to the varying resistance profiles observed between gram-negative and gram-positive bacteria.

2.2.1 Antibiotic Grouping by mechanisms	
Cell wall synthesis Inhibitors	Penicillins, Cephalosporins, Vancomycin, Beta-lactamase inhibitors, Carbapenems, Aztreonam, Polymyxin, Bacitracin.
Protein Synthesis Inhibitors	Inhibit 30s Subunit: Aminoglycosides (gentamicin). Inhibit 50s Subunit: Macrolides, Chloramphenicol, Clindamycin, Linezolid, Streptogramins.
DNA Synthesis Inhibitors	Fluoroquinolones, Metronidazole
RNA synthesis Inhibitors	Rifampin
Mycolic acid synthesis Inhibitors	Isoniazid
Folic Acid synthesis inhibitors	Sulfonamides, Trimethoprim

Table 2.1: Classification of antibiotics based on mechanism of action (Kapoor *et al.*, 2017)

2.2.2 Antibiotic Grouping by structure	
Penicillins	1. Natural: Penicillin G, Penicillin-VK 2. Penicillinase Resistant: Methicillin, Nafcillin, Oxacillin etc. 3. Aminopenicillins: Ampicillin
Fluoroquinolones	First generation: Norfloxacin, Ofloxacin, Ciprofloxacin, Pefloxacin Second generation: Levofloxacin, Moxifloxacin, Lomefloxacin, Gemifloxacin, Sparfloxacin, Prulifloxacin
Aminoglycosides	Streptomycin, Gentamycin, Kanamycin, Tobramycin, Amikacin, Sisomicin, Netilmicin
Monobactams	Aztreonam
Carbapenems	Imipenem, Meropenem, Faropenem, Doripenem
Macrolides	Azithromycin, Clarithromycin, Dirithromycin, Erythromycin, Clindamycin, Roxithromycin
Others	Clindamycin, Vancomycin, Linezolid, Rifamycin, Tetracycline, Trimethoprim/Sulfamethoxazole, Chloramphenicol etc.

Table 2.2: Classification of antibiotics based on structure (Kapoor *et al.*, 2017)

2.3 ARB and priority pathogens: Global and Indian scenario

2.3.1 WHO priority pathogens

In 2017, the World Health Organization (WHO) released a list of "priority pathogens," which consists of 12 bacterial families that pose a significant risk to human health due to antibiotic resistance. The list is categorized into three priority levels, determined by the urgency and critical need for the development of new antibiotics.

Priority 1: CRITICAL	• *Acinetobacter baumannii*, carbapenem-resistant • *Pseudomonas aeruginosa*, carbapenem-resistant • *Enterobacteriaceae*, carbapenem-resistant, ESBL-producing
Priority 2: HIGH	• *Enterococcus faecium*, vancomycin-resistant • *Staphylococcus aureus*, methicillin-resistant, vancomycin-intermediate, and resistant • *Helicobacter pylori*, clarithromycin-resistant • *Campylobacter spp.*, fluoroquinolone-resistant • *Salmonellae*, fluoroquinolone-resistant • *Neisseria gonorrhoeae*, cephalosporin-resistant, fluoroquinolone-resistant
Priority 3: MEDIUM	• *Streptococcus pneumoniae*, penicillin-non-susceptible • *Haemophilus influenzae*, ampicillin-resistant • *Shigella spp.*, fluoroquinolone-resistant

Table 2.3: WHO global priority pathogens list of antibiotic-resistant bacteria

2.3.2 XDR *Escherichia coli*

E. coli is a type of gram-negative bacteria that belongs to the group of facultative anaerobes. While E. coli commonly exists as a harmless commensal bacterium in the human body, certain strains can also be pathogenic. Pathogenic E. coli strains have the ability to produce various toxins, including enterohemorrhagic verotoxin (also known

as Shiga-like toxin). This toxin can lead to severe conditions such as hemolytic-uremic syndrome and renal failure, which can be life-threatening (Mueller, 2023).

"*E. coli* has been one of the most widely antibiotic susceptible of the *Enterobacteriaceae f*amily. Recently, though, horizontal gene transfer has allowed for the rise of highly resistant strains. The increasing resistance of E. coli is a cause for concern due to its prevalence as a leading cause of gram-negative bacterial infections in humans. Of particular worry is the emergence of strains that possess ESBLs, which provide resistance against third-generation cephalosporin antibiotics. This resistance trend has been steadily increasing in India. (AMR Surveillance Network, Indian Council of Medical Research, 2020)."

*E. col*i strains on different continents have acquired the New Delhi Metallo-βlactamase-1 (NDM-1) enzyme from K. pneumoniae. This enzyme provides resistance to a wide range of β-lactam antibiotics, including carbapenems, except for the monobactam aztreonam. This finding was reported by Khan et al. in 2017.

2.3.3 MDR and Pan-drug-resistant *Klebsiella pneumoniae*

K. pneumoniae is a type of gram-negative bacterium that is facultative anaerobic. It is primarily an opportunistic pathogen, capable of causing infections in both hospital settings (nosocomial) and within the community.

This species tends to acquire multiple drug-resistant determinants, including a wide range of β-lactamases. Of particular concern are extended-spectrum β-lactamases (ESBLs), Klebsiella pneumoniae carbapenemase (KPC), and, more recently, New Delhi Metallo-β-lactamase-1 (NDM-1). These enzymes have caused several epidemics and, even more alarmingly, have the ability to spread to other bacterial species. Carbapenem resistance poses a significant challenge as carbapenems were previously considered highly effective against most β-lactamases and were commonly used as a last-resort treatment for severe gram-negative infections. However, with the emergence of KPC and other mechanisms, carbapenem resistance has become increasingly prevalent. In some cases, pan-resistant strains, which are resistant to multiple classes of antibiotics, have been reported (Fils *et al.*, 2021).

2.3.4 Indian scenario

According to the Annual Report on Antimicrobial Surveillance in India conducted by ICMR in 2021 following AMR trends and patterns from the country were revealed:

- "Imipenem susceptibility of *E. coli* has dropped steadily from 86% in 2016 to 64% in 2021 and that of *Klebsiella pneumoniae* dropped steadily from 65% in 2016 to 45% in 2020 and was at 43% for the year 2021.
- Resistance to carbapenems in *Acinetobacter baumannii* was recorded as 87.5% in the year 2021, limiting the availability of available treatment options.
- In *Staphylococcus aureus*, susceptibility to erythromycin, clindamycin, ciprofloxacin, co-trimoxazole and high-level mupirocin was more evident in MSSA when compared to MRSA.
- Among diarrheal pathogens (Diarrheagenic *E. coli*, *Shigella spp*. and *Salmonella*) norfloxacin susceptibility was poor."

2.4 Methods for Antimicrobial Resistance Diagnostics

Performing antimicrobial susceptibility testing (AST) on bacterial pathogens is a crucial task in order to assess their susceptibility to antimicrobial agents and identify any potential drug resistance. This testing helps guide appropriate antibiotic treatment decisions and assists in monitoring the emergence and spread of antimicrobial resistance.

2.4.1 Phenotypic Methods

Culture-based methods are commonly used for phenotypic resistance detection, where bacterial growth is evaluated in the presence of antibiotics. These methods can be divided into two categories: "manual" and "automated".

"In manual methods, bacterial cultures are grown on agar plates with antibiotic discs, and the growth inhibition zones around the discs are measured. Manual tests include agar dilution, gradient test, disk diffusion, and broth microdilution antimicrobial susceptibility testing methods."

"Automated methods utilize specialized equipment and systems to automate the process of inoculating bacterial cultures and measuring their growth in the presence of antibiotics. The automated commercial platforms (VITEK®2 COMPACT, Sensititre™

ARIS™ 2X, and Alfred 60AST system) use some of the methods. The above-mentioned technologies offer qualitative and quantitative data for the strain under investigation."

"The European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) are two prominent organizations that play a key role in the regular revision and update of antimicrobial susceptibility testing (AST) standards. These organizations provide guidelines and recommendations for performing AST, including the selection of appropriate antimicrobial agents, interpretation of test results, and reporting of susceptibility or resistance. Their efforts ensure standardized and reliable methods for AST in clinical laboratories, promoting consistent and accurate assessment of antimicrobial susceptibility patterns in bacterial pathogens (Kaprou, 2021)."

2.4.2 Community Genomics-Based Methods

Molecular-based assays, which focus on the detection of ARGs, provide several advantages compared to phenotypic assays. These molecular assays utilize techniques such as polymerase chain reaction (PCR) or DNA sequencing to directly detect and characterize specific ARGs.

One advantage is the ability to perform multiplex targeting, where multiple ARGs can be simultaneously analysed in a single assay. This allows for efficient screening of multiple resistance genes in a single test, saving time and resources.

In cases where susceptibility breakpoints have not been established for certain taxonomic units, molecular-based methods serve as a viable alternative. Susceptibility breakpoints are specific thresholds that define whether a particular bacterial strain is categorized as susceptible, intermediate, or resistant to a given antimicrobial agent. However, establishing susceptibility breakpoints can be challenging for less common or emerging pathogens, as well as for novel antimicrobial agents. In such situations, molecular-based methods offer an alternative approach to determine the presence or absence of resistance genes directly, without relying on predefined breakpoints.

Furthermore, molecular-based assays offer more precise characterization and detection of AMR genes. They can provide detailed information about the specific genetic variants and mechanisms of resistance present in a bacterial sample. This level of

specificity enables a better understanding of the resistance profile and can assist in guiding appropriate treatment decisions.

Polymerase chain reaction (PCR)-based genotypic techniques, including both conventional PCR and real-time PCR, are widely available as commercial assays and automated platforms. These techniques utilize the principles of DNA amplification to detect specific DNA sequences associated with a particular pathogen and its drug susceptibility or resistance.

Conventional PCR involves a series of temperature cycles that enable the amplification of a targeted DNA sequence. By designing primers specific to the genetic markers or resistance genes of interest, PCR can selectively amplify those sequences if present in the sample. The amplified DNA products can then be visualized using gel electrophoresis to determine the presence or absence of the target sequence.

Real-time PCR, also known as quantitative PCR (qPCR), allows for the detection and quantification of DNA amplification in real-time. This technique utilizes fluorescent probes or dyes that bind to the amplification products during the PCR reaction. As the amplification progresses, the increase in fluorescence is measured in real-time, providing quantitative information about the initial amount of the target DNA sequence.

These PCR-based genotypic techniques provide a rapid and specific means of detecting the presence of specific pathogens and determining their drug susceptibility or resistance profiles. By targeting known genetic markers or resistance genes, these assays enable the identification of pathogens and their associated resistance mechanisms with high sensitivity and specificity (Vasala *et al.*, 2020).

2.4.3 Next-generation sequencing (NGS)

The COVID-19 pandemic has highlighted the usefulness of next-generation sequencing (NGS) technologies in detecting and monitoring the spread of SARS-CoV-2 variants. As a result, there is growing recognition of the potential of NGS technologies to support the management of infectious diseases.

Given the success of NGS in the context of COVID-19, there is an opportunity to explore its application in addressing other ongoing and future threats, such as AMR. NGS technologies can provide valuable insights into the genetic makeup and diversity of microbial populations, including the presence of resistance genes and their

transmission patterns. By sequencing the genomes of pathogens, it becomes possible to understand their resistance mechanisms and track the emergence and spread of resistant strains

The adoption of NGS for AMR surveillance and management can offer several advantages. It allows for comprehensive profiling of microbial communities, enabling the detection of both known and novel resistance genes. Additionally, NGS can provide information about the genetic context of resistance genes, including their association with mobile genetic elements and potential transfer between different bacteria species.

The use of NGS in LMICs may present challenges due to limited resources and infrastructure. However, as technology advances and becomes more accessible, there is potential for its wider implementation and integration into existing AMR surveillance and control programs. Collaborative efforts between HICs and LMICs can help facilitate knowledge sharing, capacity building, and the development of cost-effective approaches to leverage NGS technologies in mitigating AMR threats.

Overall, the success of NGS in the context of the COVID-19 pandemic has demonstrated its value in infectious disease management. Expanding its application to address AMR can enhance our understanding of resistance mechanisms, aid in surveillance efforts, and support the development of effective strategies to combat antimicrobial resistance. (John, 2021).

Whole Genome Sequencing

Whole Genome Sequencing (WGS) is a powerful NGS approach that provides the complete nucleotide sequence of an organism's genome. It offers the highest resolution information available, making it a valuable tool in various applications, including pathogen identification, relatedness analysis, determination of virulence factors, and assessment of drug resistance or susceptibility.

One of the key advantages of WGS is its ability to offer greater sensitivity and specificity compared to traditional phenotypic and molecular methods. By sequencing the entire genome, WGS can provide a comprehensive understanding of the genetic composition of a pathogen. It enables precise identification of the organism and can reveal its relatedness to other pathogens, shedding light on the epidemiology and transmission patterns.

In terms of drug resistance, WGS allows for the detection and characterization of resistance genes at a level of detail that is not easily achievable with other methods. It provides insights into the genetic mechanisms underlying drug resistance, including the identification of specific mutations or genetic elements associated with resistance. This information can help guide treatment decisions by informing clinicians about the most appropriate antibiotics to use.

WGS offers a multiplexed and comprehensive approach, as it can provide all the relevant information—pathogen identification, relatedness, virulence, and drug resistance—in a single assay. This reduces the need for multiple separate tests and streamlines the diagnostic process.

With its high resolution and comprehensive nature, WGS is being proposed as a potential replacement for many of the currently used phenotypic and molecular methods for various pathogens. It offers a more accurate and detailed assessment of pathogens and their characteristics, paving the way for more targeted and effective interventions in infectious disease management.

However, it's worth noting that the widespread adoption of WGS as a routine diagnostic tool may still face challenges in terms of cost, infrastructure requirements, and data analysis capabilities, particularly in resource-limited settings. Nonetheless, ongoing advancements in technology and decreasing costs are making WGS increasingly accessible and feasible for broader implementation in clinical and public health settings. (Vasala *et al.*, 2020).

NGS Workflow

"Key steps in a basic NGS workflow can be summarised as follows:

- **Sample collection and preparation** - Includes the steps from the collection of a sample through to the storage and transportation of that sample, isolation of the pathogen using a culture-based method if appropriate, to the extraction of DNA (or RNA) prior to further processing. For certain sequencing approaches, extracted DNA may undergo further amplification.
- **Library preparation** - Transforms the retained nucleic acid portion of a collected sample into a prepared sample library ready for sequencing. This may include fragmenting or size selection of nucleic acids (dependent upon

application), the addition of sequencing adapters and quantification and quality control of resulting libraries.

- **Sequencing** – The process by which the sequence of bases in a series of nucleic acids is detected by one of several methods to provide readable data – raw sequence reads. This process covers the entry of a prepared sample library into a sequencing system to retrieve raw sequence information. Different sequencing approaches will capture different amounts of sequencing data. Broadly, these sequencing approaches are described as targeted, whole genome or metagenomic.
- **Bioinformatic analysis** - This includes the processing and conversion of raw data that is produced during sequencing into one of several formats that is suitable for ongoing analysis or interpretation.

Bioinformatics analysis for WGS data has several key stages, including:

- Quality control
- Assembly
- Sequence annotation
- Comparison of genomes
- Confirmation of species identify
- Subtyping of isolates
- Identification of genetic determinants of AMR"

2.4.4 Metagenomics methods

Metagenomics is a scientific approach that involves the analysis and characterization of all the DNA present in a specific environment. This DNA can originate from organisms present in the environment or from free-floating DNA molecules. Metagenomics has emerged as a valuable tool for studying microbial communities and overcoming the limitations of traditional culture-based methods in detecting uncultivable or culture-resistant microorganisms (Liu, 2022).

Traditional methods of studying microorganisms rely on culturing them in the laboratory, which often fails to capture the full diversity of microbial life present in complex environments. Many microorganisms are difficult to cultivate or have specific growth requirements that are challenging to replicate in the lab. Metagenomics, on the other hand, allows for the direct extraction and analysis of genetic material from

environmental samples, providing a more comprehensive view of microbial diversity. By analysing the collective genetic material, including both the DNA from known organisms and the previously undiscovered DNA fragments, metagenomics can provide insights into the composition and functional potential of microbial communities. It enables the identification and characterization of microorganisms that cannot be easily cultured, helping to uncover new species, genes, and metabolic pathways (Navgire, 2022).

Metagenomics has found applications in various fields, including environmental microbiology, human health, agriculture, and biotechnology. It has contributed to our understanding of microbial ecosystems, microbial interactions, and the role of microorganisms in environmental processes. Additionally, metagenomics has been used to investigate the human microbiome, assess the impact of microbial communities on human health, and identify potential disease-causing microorganisms (Prayogo, 2020).

Metagenomics-based analysis

"Metagenomics employs two primary approaches for analysis: sequence-driven and function-driven. Both approaches rely on next-generation sequencing techniques, which have been developed by various commercial organizations and are operated on different sequencing platforms such as Illumina/Solexa, 454/Roche, Ion PGM from Ion Torrent, AB SOLiD System, and Oxford Nanopore MinION (Gupta, 2019)."

- **Sequence-driven metagenomics**: This approach focuses on sequencing and analyzing the genetic material present in a given environmental sample. It involves the direct sequencing of DNA or RNA extracted from the sample, without prior knowledge of the specific organisms or genes of interest. The sequencing platforms mentioned earlier, such as Illumina/Solexa and 454/Roche, are commonly used for high-throughput sequencing of metagenomic samples. The obtained sequence data is then processed, assembled, and compared against existing databases to identify known organisms and infer the presence of novel species or genetic variants (Zhang, 2021).
- **Function-driven metagenomics**: In this approach, the emphasis is on identifying and characterizing specific functions or traits encoded within the

metagenomic data. Rather than focusing solely on sequence information, functional metagenomics aims to understand the functional potential of the microbial community by assessing the genes and metabolic pathways present. This is typically achieved through the construction of metagenomic libraries, where large fragments of DNA from the environmental sample are cloned into host organisms (e.g., bacteria) for expression and subsequent functional screening. The libraries are screened for specific activities of interest (Ngara, 2018).

Both sequence-driven and function-driven metagenomics have their own advantages and applications. Sequence-driven metagenomics provides a broader view of the microbial community composition and can reveal novel species or genetic variations. Function-driven metagenomics, on the other hand, allows for the identification and exploration of specific functional capabilities encoded within the metagenome (Zhang, 2021).

The choice of sequencing platform depends on factors such as the desired sequencing depth, read length, cost, and available resources. Different platforms offer varying capabilities in terms of throughput, accuracy, and sequencing technology (Amarsinghe, 2020).

Metagenomics Workflow

Metagenomics for antimicrobial resistance (AMR) surveillance follows a series of steps:

- **Sample collection and processing**: Samples are collected from the target environment, such as clinical specimens, wastewater, or environmental sources. The samples undergo processing to extract the genetic material (DNA or RNA) present in the microbial community.
- **Library preparation**: The extracted genetic material is converted into a sequencing library through various techniques, such as fragmentation, adapter ligation, and amplification. Different library preparation methods can be employed, depending on the sequencing platform and the specific objectives of the study. Methods aiming to identify the genomic and taxonomic context of AMR genes and/or discover new microorganisms require a higher level of sensitivity and therefore necessitate greater sequencing depth compared to

methods focused on identifying known pathogens or AMR genes in the sample (Franklin, 2021).

- **DNA Sequencing**: The prepared library is then subjected to next-generation sequencing using the selected sequencing platform. If the goal is to obtain more detailed information about the genomic and taxonomic context of the sequenced microbes, an assembly and binning approach can be employed. This approach involves assigning reads to contigs, which are overlapping DNA sequences that can be assembled to reconstruct larger DNA regions or partial/whole pathogen genomes. By using this approach, it becomes possible to determine which species of microbes are associated with specific AMR genes, locate the AMR genes on the genome, and identify multi-drug resistance in pathogens. In addition to providing greater genomic context, this approach enhances the accuracy of pathogen identification, reducing the risk of false positives, and enabling the detection of novel taxa (Abreu, 2020).
- **Annotation**: Metagenomic sequence data annotation involves two main steps. Firstly, the identification of features of interest (genes) through feature prediction, and secondly, the assignment of putative gene functions and taxonomic neighbours through functional annotation. Currently, metagenomic annotation relies on classifying sequences by comparing them to known functions or taxonomic units using homology searches against annotated data. In small datasets, manual curation can be employed to improve the accuracy of automated annotation. However, metagenomic datasets are typically large, making manual annotation impractical. Automated annotation methods, such as running a BLASTX similarity search, need to be both accurate and computationally efficient. To provide functional context to metagenomic datasets, various reference databases are available, including KEGG (Kanehisa *et al.*, 2004), eggnog (Muller *et al.*, 2010), COG/KOG (Tatusov et al., 2003), PFAM (Finn et al., 2010), and TIGRFAM (Selengut *et al.*, 2007). However, no single reference database covers all biological functions. Therefore, it is important to have the ability to visualize and merge the interpretations from multiple database searches within a unified framework, as seen in the latest versions of MG-RAST and IMG/M.

- **Bioinformatics analyses**: The assembled contigs and raw sequencing data are further analyzed using bioinformatics tools and pipelines. This includes various steps such as taxonomic classification to identify the microorganisms present in the sample, functional annotation to assess the presence of AMR genes and other functional elements, and comparative analysis to evaluate the relatedness of sequences to known AMR genes or reference genomes. Additionally, data visualization and statistical analyses can be performed to interpret and summarize the results.

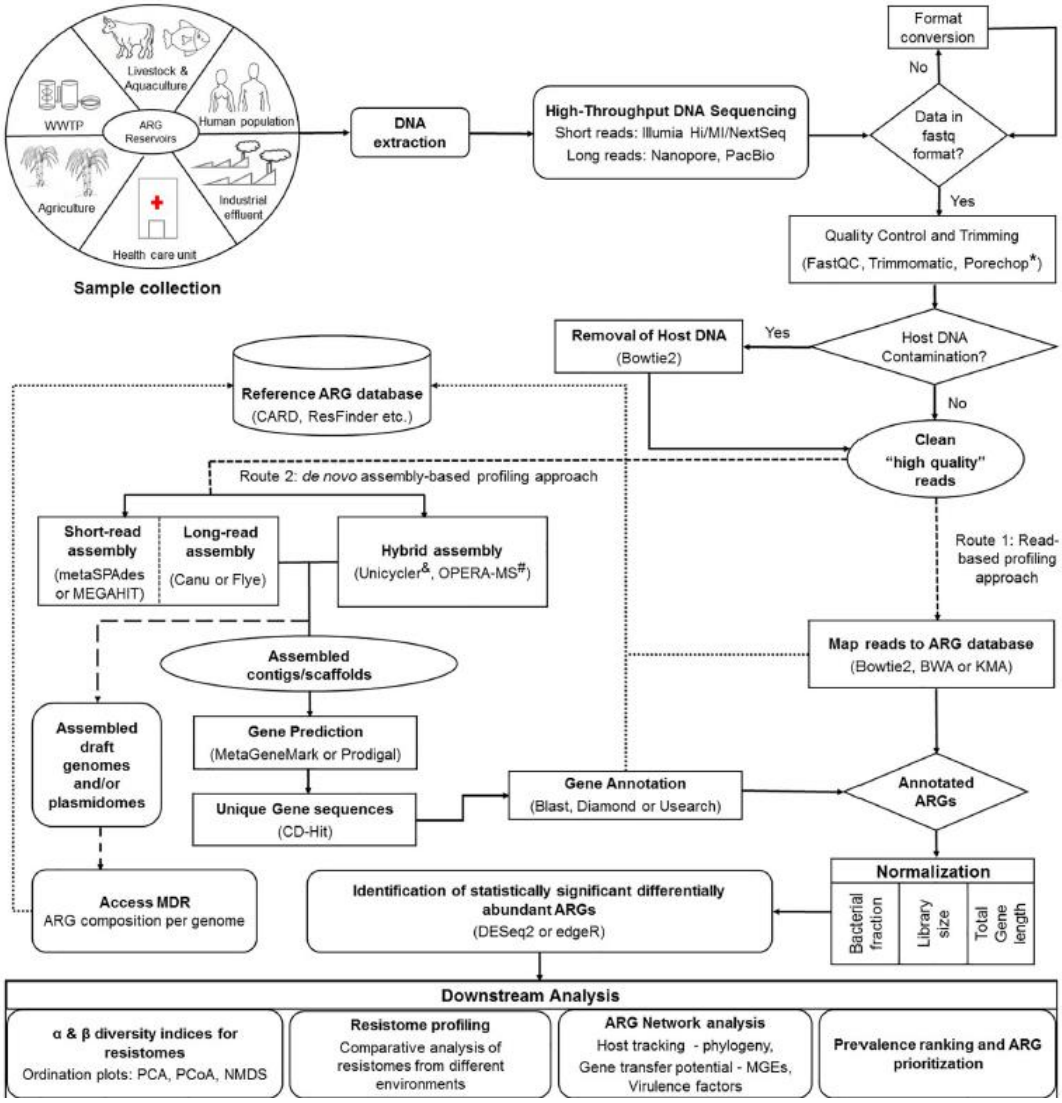


Fig. 2.2: "A suggested workflow for determining the scope and distribution of resistome in complex environments using genomic and/or metagenomic data (Gupta, 2020)"

The output of the metagenomic analysis provides valuable information for AMR surveillance purposes. It can include the identification of specific microbes, their genetic characteristics, and the presence of AMR genes or genetic variants of interest. Depending on the depth of sequencing and the quality of the data, it may be possible to achieve partial or even whole genome assembly of the microbial species present in the sample (Pillay, 2022).

This information obtained from metagenomics can be used to inform AMR surveillance efforts, track the prevalence and dissemination of AMR genes and resistant microbes, study their evolution and transmission dynamics, and guide interventions and policies to combat AMR effectively (Cave, 2021).

2.5 Computer-aided Drug Discovery

The scientific community has dedicated significant effort to studying ligand-protein interactions, which play crucial roles in various biological processes and have important implications for pharmaceutical research. Over the years, several theories have been proposed to understand the binding phenomenon, with a growing focus on considering the flexibility of both the ligand and protein molecules involved. This emphasis on flexibility has provided valuable insights into the complexities of ligand-protein interactions (Fu, 2018).

2.5.1 Theories involved

Emil Fischer proposed the "lock-key" model in 1894 to explain enzyme specificity, where the ligand rigidly recognizes and occupies the protein binding site due to their complementary shapes. However, this model could not account for enzyme non-competitive inhibition or allosteric modulation.

In 1958, Koshland introduced the "induced-fit" theory, suggesting that ligands can induce conformational changes in the protein, optimizing ligand-protein interactions. Subsequent research revealed that proteins exist as an ensemble of conformations, described by an energy landscape, and ligands preferentially bind to one of these conformations. This interpretation, known as "conformational selection," explains that ligands stabilize a specific protein conformation, shifting the equilibrium of protein populations. These two theories, although seemingly contrasting, have different ranges

of applicability and describe the binding process with different chronological sequences of events.

"Later works suggested that proteins naturally exist as an ensemble of conformations (Monod et al., 1965), described by an energy landscape (Frauenfelder et al., 1991), and ligands preferentially bind to one of them (Austin et al., 1975; Foote and Milstein, 1994). According to this interpretation of binding, known as "conformational selection," the ligand stabilizes one of the protein conformations with a consequent shift of the protein population equilibrium (Kumar et al., 2000). These two apparently contrasting theories have simply different ranges of applicability, and the descriptions they provide of molecular binding differ for the chronological sequence of events in which the binding process is decomposed (Kobilka and Deupi, 2007; Okazaki and Takada, 2008; Zhou, 2010). New theories are emerging, making a compromise between the ones: according to the extended conformational selection model, for example, the conformational selection is followed by a conformational adjustment (induced fit) (Csermely et al., 2010)."

2.5.2 Molecular docking

Molecular docking is a widely used virtual screening method, particularly when the three-dimensional structure of the target protein is known. It allows for the prediction of the binding affinity between a ligand and a protein, as well as the structure of the resulting protein-ligand complex. This information is valuable for lead optimization in drug discovery. Molecular docking has been employed for over three decades and has played a significant role in the discovery and development of numerous new drugs. The drug discovery process typically involves several stages, including target selection, hit identification, lead optimization, and preclinical and clinical studies (Torres, 2019).

The initial step in molecular docking involves preparing the protein structure. A commonly used approach is to remove solvent molecules, ions, and other small molecular ligands, resulting in an empty binding pocket that is suitable for docking. This simplifies the docking process by focusing solely on the interaction between the protein and the ligand of interest (Azad, 2023).

The 3D structure of a protein-ligand complex offers valuable atomic-level insights into the binding mechanism between the ligand and the target protein. This information is particularly crucial for lead optimization in drug discovery. However, it is important to

note that the docked binding conformation of the ligand may differ from the experimentally determined structure, especially when the ligand exhibits high flexibility and can adopt multiple conformations with small energy barriers between them (Pinzi, 2019).

The therapeutic efficacy of a drug can be enhanced by having a higher binding affinity and a longer residence time in the binding pocket of the target protein. The binding affinity is quantitatively described by the Gibbs free energy (ΔG), which is the sum of the binding enthalpy (ΔH) and the entropy change (ΔS). A stronger binding affinity, indicated by a more negative ΔG value, suggests a tighter interaction between the drug and the target protein. Additionally, a longer residence time implies that the drug remains bound to the protein for an extended period, allowing for sustained pharmacological activity (Decherchi, 2020).

The selection and preparation of a structural model for the targeted binding site play a crucial role in the molecular docking process. Experimentally determined structures, obtained through techniques such as X-ray crystallography or nuclear magnetic resonance (NMR), are typically preferred due to their high accuracy. However, the number of proteins with experimentally determined structures is limited compared to the increasing number of proteins that are of pharmaceutical interest.

To address this gap, homology modeling has gained popularity. Homology modeling involves predicting the three-dimensional structure of a protein by utilizing the known structure of a related protein as a template. This approach relies on the assumption that proteins with similar sequences share similar structures. Homology modeling allows researchers to generate structural models for target proteins based on their sequence similarity to proteins with known structures, enabling a wider range of proteins to be investigated in molecular docking studies (Salo-Ahen, 2020).

In order to efficiently screen large databases of potential ligands and reduce the computational workload of docking and scoring steps, three-dimensional filter functions have been incorporated in addition to the traditional one- or two-dimensional filters like the rule-of-five. These three-dimensional filters consider the spatial arrangement and shape of molecules to identify compounds that are more likely to bind to the target protein. Three-dimensional filter functions utilize various descriptors and algorithms to assess the molecular properties and compatibility with the binding site.

These filters can evaluate parameters such as molecular size, shape, hydrophobicity, hydrogen bonding potential, and electrostatic properties. By applying these filters, molecules that do not meet specific geometric or physicochemical criteria can be excluded from further docking and scoring calculations, reducing the number of compounds that need to be evaluated. The implementation of three-dimensional filters helps prioritize potential ligands that are more likely to have favourable binding interactions with the target protein, improving the efficiency of the virtual screening process and focusing computational resources on the most promising candidates (Zhang, 2022).

To maximize the utilization of limited computational resources, several scoring functions have been developed for molecular docking. These scoring functions aim to provide rapid predictions of the binding strength between a chemical compound and a target protein or macromolecule. The scoring functions evaluate and assign a numerical value or score to each docked pose or complex based on various factors, including energetic terms, geometric complementarity, and intermolecular interactions (Pellicani, 2023).

The scoring functions typically incorporate terms that assess the potential energy changes associated with the binding process, such as van der Waals interactions, electrostatic interactions, hydrogen bonding, and desolvation effects. These energetic terms capture the favourable and unfavourable contributions to the binding affinity and help estimate the binding free energy or binding affinity of the ligand-protein complex.

Different scoring functions may utilize different algorithms, weighting schemes, or empirical parameters to calculate the final score. Some scoring functions are knowledge-based, derived from statistical analysis of known ligand-protein complexes, while others are physics-based, utilizing force fields and energy calculations. Some scoring functions also consider additional factors, such as protein flexibility or ligand strain, to improve the accuracy of the predictions.

The ultimate goal of these scoring functions is to rank and prioritize the docked poses or compounds based on their predicted binding affinities. By rapidly estimating the binding strength, scoring functions help in the identification of potential lead compounds for further optimization and drug discovery efforts, thereby reducing the number of compounds that need to be experimentally tested (Guedes, 2018).

2.6 Plant-derived compounds: Potential antibacterial agents

Herbal medicine has been utilized since ancient times as a fundamental approach to treating various medical conditions such as colds, diarrhoea, dental problems, and labour pain. These traditional remedies have remained a preferred choice for patients due to their perceived lower incidence of side effects and their affordability, particularly in underdeveloped countries. Although there are approximately 17,000 identified plant species, only around 3,000 of them are currently used in medical applications. However, according to the World Health Organization, a significant 80% of the global population still relies on folk medicine to meet their healthcare needs. This emphasizes the ongoing importance of exploring medicinal plants for their potential therapeutic properties. Plants are extensively investigated as a valuable source for developing new therapeutic agents, and there is a growing interest in exploring the use of crude plant extracts for the treatment of various diseases. The search for medicinal plants with promising activity continues to hold significant value in the field of healthcare and drug discovery (Sarecka-Hujar, 2022).

Numerous studies have focused on exploring new alternative medications to address the challenge of drug resistance. Nature, particularly plants, has been recognized as a valuable source for drug discovery. Scientists have been drawn to the phytochemicals found in plants due to their diverse structures, lower incidence of side effects, and greater acceptability among individuals. Many of these phytochemicals have exhibited antimicrobial activity, making them of particular interest in the search for new drugs. In fact, it is estimated that around 25% of the existing pharmacopoeia, which refers to the collection of drugs and drug formulations, consists of compounds derived from plants. This highlights the significant contribution of plant-derived compounds to the development of medicines (Jubair, 2021; Nwonuma, 2019).

Plant-derived medicines play a crucial role in the healthcare systems of developing countries, with approximately 80% of these nations relying on such medicines as their primary treatment options. Plants continue to be at the heart of various traditional medical practices, including Ayurveda (the traditional Indian medicinal system) and Traditional Chinese Medicine (TCM). These systems of medicine have a long history of utilizing plant-based remedies and emphasize the holistic approach to health and well-being. The reliance on plant-derived medicines in these traditions underscores

their significance in providing accessible and culturally relevant healthcare solutions to a significant portion of the global population (Prasathkumar, 2021).

2.6.1 Plant metabolites

Plants synthesize two main types of metabolites: primary metabolites and secondary metabolites. Primary metabolites are crucial for the plant's survival and are involved in essential processes such as nutrition and reproduction. They are produced through pathways such as glycolysis, the shikimate pathway, and the tricarboxylic acid cycle.

On the other hand, secondary metabolites are synthesized in response to plant interactions with the environment. They are produced at various stages within the primary metabolic pathways and serve as a defence mechanism against biotic stresses (such as bacteria, fungi, insects, and diseases) as well as abiotic stresses (such as injuries, temperature changes, and moisture fluctuations) (Dhaniaputri, 2022).

Enzymatic activities play a significant role in the production of secondary metabolites from primary metabolites, leading to the generation of diverse compounds that aid in plant adaptation and defence. These secondary metabolites have potential applications in various fields, including medicine, agriculture, and industry, due to their bioactive properties and therapeutic potential (Pott, 2019).

Plant-derived compounds are secondary metabolites that are produced as part of plant metabolism and can be found in various plant parts such as roots, leaves, bark, flowers, and seeds. These compounds exhibit a wide range of structural diversity. They have garnered significant attention in the pharmaceutical industry for their potential to enhance the biological activity of existing antibiotics or serve as a source for new antimicrobial agents that can target multidrug-resistant (MDR) bacteria and other pathogens (Hussein, 2018).

2.6.2 Classes of secondary metabolites

Plant phytochemicals can be categorized into different classes based on their biosynthetic origin. These classes include terpenoids, polyketides, phenylpropanoids, and alkaloids (Eljounaidi, 2020).

- Terpenoids are derived from the mevalonate or methylerythritol phosphate pathways and encompass compounds like essential oils, steroids, and carotenoids.
- Polyketides are synthesized through the polyketide synthase pathway and include compounds such as anthraquinones and polyphenols.
- Phenylpropanoids are derived from the shikimate and phenylpropanoid pathways and consist of compounds like flavonoids and lignans.
- Alkaloids are a diverse group of nitrogen-containing compounds that are synthesized through various pathways and include compounds such as alkaloids, alkaloids, and alkaloids.

The structural diversity and bioactive properties of these plant-derived compounds make them promising candidates for drug development and the discovery of new therapeutic agents.

2.6.3 PDA: Mechanisms of action

There are various reports which address the bioactivities of plant antimicrobials against various mechanisms that contribute to bacterial resistance to multiple drugs (Vaou, 2021; Cheesman, 2017). These mechanisms include:

- **Inhibition of biofilm formation**: Biofilms are communities of bacteria that adhere to surfaces and form a protective matrix. They are known to enhance bacterial resistance to antibiotics. Plant antimicrobials have shown activity in inhibiting the formation and development of biofilms, preventing bacteria from establishing a protective environment and making them more susceptible to treatment (Roy, 2018).
- **Efflux-pump inhibition**: Efflux pumps are transporters present in bacterial cells that actively pump out antibiotics, reducing their intracellular concentration and rendering the bacteria resistant. Plant antimicrobials have demonstrated the ability to inhibit these efflux pumps, thereby preventing the bacteria from expelling antibiotics and increasing their susceptibility to drug treatment (Sharma, 2019).
- **Attenuation of bacterial virulence**: Bacterial virulence refers to the ability of bacteria to cause disease and evade the immune system. Some plant antimicrobials have been found to interfere with the virulence factors of

bacteria, such as adhesion, toxin production, and biofilm formation. By targeting these virulence mechanisms, plant antimicrobials can reduce the pathogenicity of bacteria and enhance the effectiveness of conventional antibiotics (Tako, 2020).

These bioactivities of plant antimicrobials provide potential strategies to overcome bacterial resistance and improve the efficacy of antimicrobial therapies.

2.6.4 Screening of PDA for potential leads

The screening of crude plant extracts for synergistic interactions with antibiotics can provide valuable insights into identifying bioactive compounds that can be used in combination therapy. By testing the extracts in combination with antibiotics, researchers can identify plant compounds that enhance the antimicrobial activity of the antibiotics, potentially overcoming bacterial resistance (Vaou, 2021).

Bioinformatics tools, programs, and servers play a crucial role in this process. They assist in the identification of different active compounds from plants that possess antimicrobial potential. These tools can analyze large datasets and perform virtual screening to identify molecules with desired properties. They also enable interactive visualization and analysis of molecular structures, aiding in the understanding of the compounds' interactions with bacterial targets.

Once promising phytocompounds are identified, further steps involve their isolation and purification. This process helps obtain purified compounds that can be subjected to rigorous testing and characterization (Mahato, 2019). Molecular docking, a computational method, is often employed to study the interactions between these phytocompounds and proteins of potential multi-drug resistant bacteria. This approach provides insights into the binding modes and affinities of the compounds, guiding the design of new drugs that combine antibiotics and phytocompounds to effectively treat infectious diseases.

The integration of screening methods, bioinformatics tools, and molecular docking facilitates the discovery of novel antimicrobial phytocompounds and their potential use in combination therapies. By combining the strengths of antibiotics and plant compounds, there is a possibility of developing more effective treatment strategies against infectious diseases, particularly those caused by MDR bacteria (Miethke, 2021).

2.6.5 Antibacterial Potential of Ethnomedicinal ferns in India

Pteridophytes, also known as vascular cryptogams or ferns, are widely distributed throughout India. They can be found in various habitats, including forests, wetlands, mountains, and even urban areas. However, it is worth noting that pteridophytes are generally known to prefer shade or partially shaded environments.

These plants have adapted to thrive in shaded conditions, where they can take advantage of lower light levels. Their preference for shade is attributed to their unique morphology and physiological characteristics, such as their specialized leaves called fronds and their ability to efficiently capture and utilize light energy in low-light conditions (Nayak, 2013).

Theophrastus, an ancient Greek botanist, recognized the medicinal value of ferns in one of his books. In India, although the total number of fern species is 1,022, they have been overshadowed by the vast diversity of angiosperms, which encompasses around 15,000 species. However, ferns have played a significant role in ethnomedicine, particularly in ancient Indian medicine and among Unani physicians in India and Western Asia.

Historical records indicate that ferns were widely used in traditional medicine by the people of India and various other countries. Many fern species were employed for their therapeutic properties, and their usage was particularly focused on treating diseases caused by bacteria, including gram-positive, gram-negative, and acid-fast bacteria. The preparations derived from fern plant extracts were successfully utilized in the treatment of such diseases and were believed to possess antimicrobial properties (Nath, 2017).

While ferns have often been overshadowed by the prominence of angiosperms in the Indian context, their historical and ethnomedicinal significance should not be overlooked. The traditional use of ferns in medicine reflects the rich botanical knowledge and healing practices of ancient cultures, highlighting the potential value of these plants in the development of novel therapeutic approaches (Mandal, 2020).

The investigation of the antibiotic activity of ferns has received limited attention in terms of systematic surveys. A study conducted by Banerjee and Sen in 1980 indicates that comprehensive research on the antibiotic properties of ferns has been relatively scarce.

Christella dentata: Antibacterial potential

Christella dentata (Forssk.) & Jermya is a member of the fern family *Thelypteridaceae* and is characterized as a facultative wetland plant with a widespread distribution. It typically thrives in wet habitats such as along the banks of streams, riverbeds, swampy sites, and even under overhanging cliffs. The plant is known to be edible, as mentioned in a study by Kumar et al. in 2003, and it has been used in traditional folk medicine for the treatment of skin diseases, as reported by Kumar and Dash in 2012. However, there is limited available literature regarding the phytochemical analysis of this fern species.

According to a study conducted by Singh et al. in 2020, antimicrobial assay results indicated that root extracts of *C. dentata* have higher efficacy towards inhibiting the microorganisms such as *E. coli*, *Bacillus*, *Lactobacillus* and *Salmonella*. The investigation was based on the biochemical and antimicrobial analysis of *Christella* leaves and roots which showed strong efficacy of their extracts in biochemical as well as in antimicrobial assays. Some of the compounds under study displayed affinity towards binding to the target proteins with stable complex formation as indicated by the interaction energies and thus can be further tested experimentally for their establishment as leads for drug discovery.

3. OBJECTIVES OF THE STUDY

3. Objectives of the study

The study was based on the following objectives:

1. Metagenomic & chemical profiling of effluents and wastewater collected from selected sampling sites.
2. Isolation and characterization of individual isolates using microbiological, biochemical, and molecular techniques.
3. Antibiotic resistance profiling of isolated strains.
4. Whole genome analysis of XDR strains.
5. Identification of disease targets (proteins) of the identified leads.
6. Virtual screening of medicinal plant library to identify potent chemical pharmacophores and drug likeliness analysis.

4. MATERIALS AND METHODS

4. Materials and Methods

4.1 Sample collection

"Water quality assessment holds significant importance in the realm of environmental considerations. Regular sampling intervals are crucial for analyzing the presence of organic and potentially hazardous substances. It is essential to prioritize the examination of resistance rates among clinical isolates. However, gaining a comprehensive understanding is vital to limit the local and global dissemination of antibiotic resistance. Disregarding the pollution of the environment caused by wastewater would be unwise since the environment inevitably reciprocates. Wastewater sampling can serve as an early warning system for detecting the emergence of new or uncommon bacterial antibiotic resistance within the target population. The concept of utilizing samples from hospitals (HW) and urban wastewater (UW) to study antibiotic resistance in the corresponding human population was initially proposed by Linton et al. (1974). Their findings suggested that the bacterial flora of the normal healthy population significantly contributed to antibiotic-resistant bacteria in UW, while HW exhibited notably higher rates. A comprehensive European study on enterococci from diverse environments revealed that the bacterial population structure in wastewater samples closely resembled that of a large collection of fecal samples from individual humans, indicating that they could be treated as pooled fecal samples. Emphasizing randomized sampling strategies is essential to ensure proper statistical data analysis, mitigate sampling bias, and achieve representative samples that reflect variations in management and hygiene practices across different rural and urban regions.

During our specific study, untreated hospital wastewater and sludge were aseptically collected in sterile containers from both rural and urban areas of West Bengal, extracted directly from the primary drainage systems of the hospitals. The collected effluents were promptly transported within a time frame of 10 hours and stored at a temperature of −20 °C until further processing."

4.1.1 Study area

"Purulia, which spans latitudes ranging from 22°42′35′′ to 23°42′00′′ North and longitudes ranging from 85°49′25′′ to 86°54′37′′ East, is marked as the first site of this research. This region is characterized by a dry and arid climate, with certain areas of the Chotanagpur plateau region contributing to its warm and humid conditions. The sample collection site in Purulia was near a medical facility, specifically targeting

wastewater effluent. The climate conditions at the time of collection were documented as follows: Temperature: 42°C, relative humidity: 68%, Time of collection: 12 p.m.
The second area under investigation is Kolkata, situated in the eastern part of India. The Calcutta Municipal Corporation encompasses a total area of 185 square kilometers. Historically, a significant portion of the city consisted of marshy wetlands, with remnants still present, particularly in the eastern parts (KMDA Report 2011). The sample collection site in Kolkata focused on solid sludge and associated wastewater, collected near a large urban medical facility located in central Kolkata. The climate conditions at the time of collection were recorded as follows: Temperature: 34°C, Relative humidity: 95%, Time of collection: 11:45 am."

4.2 Evaluation of physicochemical parameters of the sampling stations

"The physicochemical parameters of the effluents were analyzed as per standardized EPA protocols (Ram 2011). The evaluation was outsourced to Envirocheck, South Dumdum, West Bengal. The parameters like Chemical oxygen demand (COD), Total organic carbon (TOC), Nitrate, Ammonia, total Kjeldahl nitrogen, total Phosphorus, and heavy metals, viz. Chromium (Cr), Mercury (Hg), Lead (Pb), Cadmium (Cd), Arsenic (Ar) were evaluated."

4.3 Culture-independent analysis: Metagenomics profiling

"Each sample's DNA was isolated using the methodology described by Bonet et al. (2014). The DNA was quantified using the Qubit dsDNA HS Assay kit (Life Tech). The concentration was evaluated using a Qubit® Fluorometer and one µl of each sample. The microbial genomic DNA concentration in hospital effluents was normalised to 10 ng/ µl."

4.3.1 Design of PCR primers with multiplexing index and Illumina sequence adapters

"The PCR library preparation of amplicons was carried out using Nextera XT Index Kit (Illumina, Inc.). The 16S Metagenomic Sequencing Library preparation protocol was followed. Primers for the amplification of the V3-V4 hyper-variable region [V3 Forward Oligo: CCTACGGGNBGCASCAG and V4 Reverse Oligo: GACTACNVGGGTATCTAATCC] of 16S rDNA gene of bacteria and Archaea were used."

4.3.2 PCR amplification of target region

"The amplification of amplicons with the Illumina adaptors was performed by i5 and i7 primers that add multiplexing index sequences as well as common adapters required for cluster generation (P5 and P7) as per the standard Illumina protocol. The purification of amplicon libraries was done by 1× AMpureXP beads and checked on Agilent DNA 1000 chip on Bioanalyzer 2100 and quantified on fluorometer by Qubit dsDNA HS Assay kit (Life Technologies). The library size of Sample LS and Sample SS was 2 million reads each."

4.3.3 Sequencing

"Genome sequencing reveals the order of DNA nucleotides (the order of As, Cs, Gs and Ts) in genome/ organisms. The sequencing of the libraries was done using the Illumina sequencing chemistry to generate ~150 Mb of data per sample. After obtaining the Qubit concentration for the library and the mean peak size from the Bioanalyzer profile, the library was loaded onto the Illumina Platform at the appropriate concentration (10–20 pM) for cluster generation and sequencing. The PCR amplicons were tagged with complementary adapter oligos on paired-end flow cells using the kit reagents. The designing of adapters was done as such to allow selective cleavage of the forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand was then used to perform sequencing from the opposite end of the fragments."

4.3.4 Bioinformatics analyses

"The quality control of raw reads was performed by using the FASTQC toolkit (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). The quality processed paired-end reads were analyzed with QIIME for a comprehensive and comparative analysis of the environmental metagenome data. QIIME data was further used to determine the gene composition and alpha rarefaction within the sample.

The Quality control parameters suggest good-quality reads. The sequence length distribution is at 250 bp, there were no ambiguous bases present in the reads and the average Phred score was >30 for all the reads."

4.3.5 16s rRNA Metagenome Analysis

"The processed paired-end reads were clustered into OTUs (Operational Taxonomic Units) by using QIIME software (qiime.org) in order to identify the microbial community profile. The OTUs were further processed for taxonomic assignment (Greengenes database), phylogenetic and diversity analysis. QIIME (an abbreviation

for Quantitative Insights into Microbial Ecology) is a bioinformatics pipeline designed for analyzing microbial communities. The software clusters the marker gene nucleotide sequences into OTUs (Operational Taxonomic Units) and taxonomically annotates the OTUs by looking for sequences similar to them on a reference taxonomic database. Rarefaction curves are necessary for estimating species richness."

4.4 Taxon set enrichment analysis

"From the metagenomics-based analysis, the generation of a predicted pathogenic load network graph is possible which considers taxon enrichment and also enables to correlation of the data with genus abundance profiles."

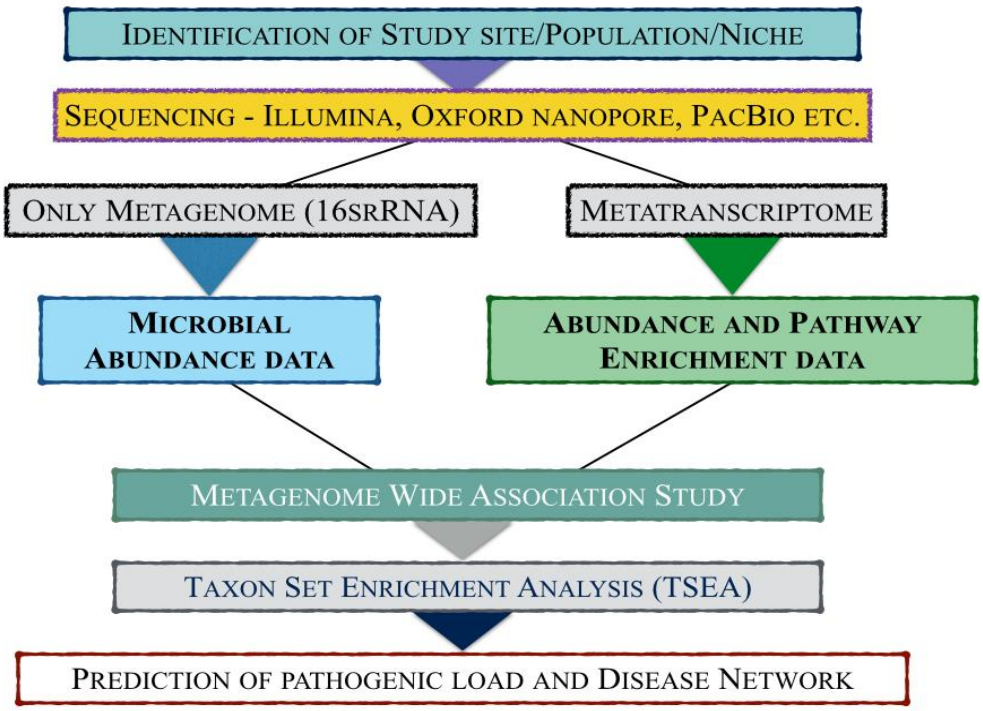


Fig. 4.1: "Proposed pipeline for detection of pathogenic load in an environmental niche"

4.5 Pathway analysis

"Pathway analysis is a computational approach used in bioinformatics and genomics to study the collective behavior of genes or proteins in biological pathways. One popular resource for pathway analysis is the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

The KEGG database is a comprehensive collection of biological pathway maps, gene catalogs, and other resources that help researchers interpret large-scale datasets. It covers various organisms and provides information on cellular processes, metabolic pathways, signaling pathways, and diseases.

In pathway analysis using the KEGG database, researchers typically start by mapping their gene or protein identifiers to the corresponding KEGG identifiers. This process is known as pathway enrichment analysis or pathway mapping. It helps identify which pathways are significantly enriched in the dataset under investigation.

Once the mapping is done, the researchers can analyze the functional and biological significance of the identified pathways. They can assess the involvement of specific pathways in a particular biological process, infer potential relationships between genes or proteins within a pathway, and gain insights into the underlying molecular mechanisms.

Pathway analysis in the KEGG database allows researchers to visualize and explore pathway maps, identify key genes or proteins within pathways, and study the interactions and relationships among them. It helps in understanding the biological context of gene expression data, identifying potential drug targets, and uncovering dysregulated pathways in diseases.

KEGG provides various tools and resources to facilitate pathway analysis, including the KEGG pathway maps, KEGG Orthology (KO) identifiers for genes and proteins, and pathway enrichment analysis tools such as KEGG Pathway Mapper and KEGG Mapper-Reconstruct Pathway."

4.6 Culture-dependent analysis: Microbiological characterization

4.6.1 Pure colony isolation

"The effluent samples were serially diluted and plated onto nutrient agar plates (pH 6.6) and were incubated overnight at 37 °C. Isolated colonies in higher dilution were observed on each type of plate. These colonies were then subcultured in nutrient agar plates for pure colony isolation and subsequently Gram stained. The colonies were observed under 100*x* Magnification of the objective lens of a Bright Field Light Microscope for morphological study. The Gram-positive and Gram-negative bacterial isolates were then presumptively identified through a series of conventional

morphological, cultural, and biochemical tests according to the criteria described in Bergey's Manual of Determinative Bacteriology."

4.6.2 Biochemical characterization

"Biochemical characterization of bacteria refers to the assay of their different enzymatic activity and production or utilization of certain biochemical substrates. Routine biochemical tests for the characterization of an unknown bacterium include the following."

4.6.2.1 Urease Test

"Many microorganisms can produce a hydrolytic enzyme urease that breaks the nitrogen and carbon bond in an amide compound like urea and results in the production of ammonia. Among the bacterial kingdom, the genus *Proteus* is the most efficient in producing this enzyme along with the other genus. Production of ammonia through urease activity would increase the pH of the media containing urea which changes the colour of the redox dye phenol red from red to pink.

$CO(NH_2)_2 + 2H_2O \rightarrow CO_2 + H_2O + 2NH_3$"

Urea Ammonia

4.6.2.2 Catalase Test

"The rhizospheric environment facilitates the growth of microaerophilic organisms. These organisms during their aerobic respiration use oxygen as a terminal electron acceptor which results in the generation of hydrogen peroxide and other toxic superoxide. To survive within this toxicity, microorganisms must produce the enzyme catalase rapidly to degrade the hydrogen peroxide produced into water and oxygen.

$2H_2O_2 \rightarrow 2H_2O + O_2$

Hence the production of the enzyme catalase is an important biochemical parameter for microaerophiles, facultative anaerobes and strict aerobes."

4.6.2.3 Nitrate Reduction Test

"Some aerobic and facultative anaerobic bacteria can utilize nitrate (NO_3^-) or sulphate (SO_4^-) as a source of oxygen under oxygen deplete conditions. These bacteria possess an enzyme Nitrate reductase that converts the nitrate into nitrite (NO_2^-) which in the

presence of sulfanilic acid and α-naphthylamine gives a characteristic red colour to the medium. A few among them can also reduce nitrite to ammonia or nitrogen. The addition of an oxidative agent in the form of zinc dust will transform these reduced nitrogenous substrates into nitrite that gives a positive signal upon reaction with sulfanilic acid and α-naphthylamine."

$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O$

Nitrate Nitrite

$NO_2^- \rightarrow NH_3$

Nitrite Ammonia

Or,

$2NO_3^- + 12H^+ + 10e^- \rightarrow N_2 + 6H_2O$

Nitrate Nitrogen

4.6.2.4 Starch Hydrolysis Test

"Monosaccharide and more preferentially glucose is the simplest form of carbohydrate or polysaccharide that can be transported into the cell and take part in cellular metabolism. Many bacteria produce extra-cellular enzymes like amylase and maltase characteristic of their biochemical property. These bacteria can hydrolyse starch, a branched polysaccharide into its building block glucose and thereby utilise this polysaccharide as their source of carbon and energy."

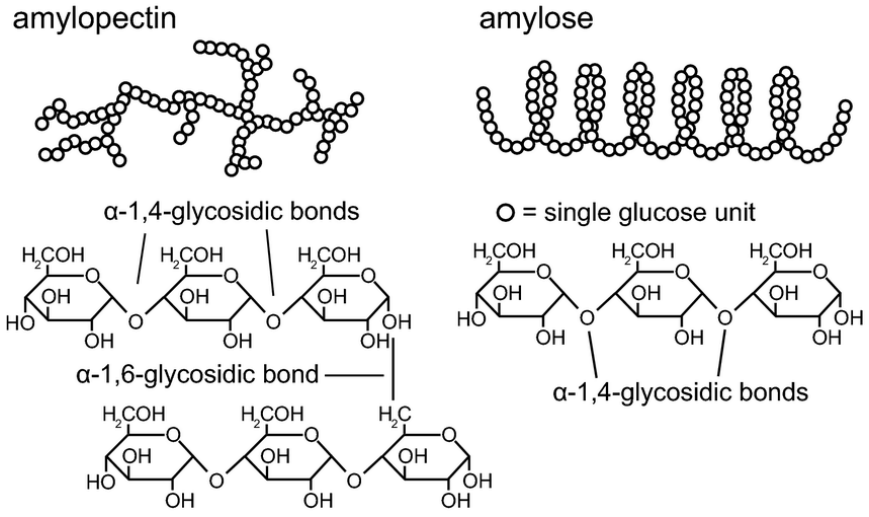


Fig. 4.2: Biochemical structure of Amylopectin and Amylose

4.6.2.5 Oxidase Test

"The terminal key enzyme of the electron transport chain of aerobic organisms is cytochrome oxidase which oxidizes the terminal cytochrome cytaa3 using oxygen as a terminal electron acceptor. P-amino dimethylaniline oxalate is an artificial substrate that act on behalf of cytochrome as a terminal electron donor and oxidized into a blackish compound."

2 reduced Cytochrome $+2H^+ +1/2\ O_2 \rightarrow$ 2 oxidized cytochrome + H_2O

4.6.2.6 Citrate utilization test

"Citrate can be utilized by some bacteria as a carbon source in the absence of glucose or lactose. It is the first major intermediate in the Kreb's cycle. The enzyme citrate breaks citrate into oxaloacetic acid and acetate. These products are then enzymatically converted to pyruvic acid and carbon-di-oxide.

Despite the production of acid, the medium becomes alkaline due to the formation of sodium carbonate upon reaction with carbon dioxide and the sodium ion present in the medium. This ultimately changes the colour of the redox dye bromothymol blue to Prussian blue."

$CO_2 + 2Na^+ + H_2O \rightarrow Na_2CO_3$

Sodium carbonate

4.6.2.7 Indole test

"The indole test is a biochemical test used to determine the ability of microorganisms, particularly bacteria, to produce indole from the amino acid tryptophan. The test is based on the activity of the enzyme tryptophanase, which breaks down tryptophan into several by-products, including indole.

The indole test is commonly performed as part of the identification and classification of bacteria, particularly in the Enterobacteriaceae family."

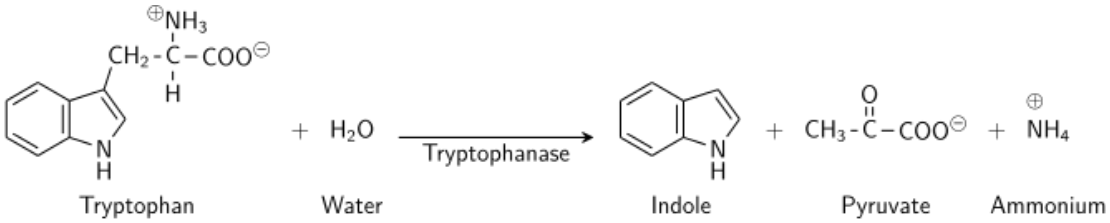


Fig. 4.3: Indole test reaction

4.6.2.8 Voges Proskauer Test

"The Voges-Proskauer (VP) test is a biochemical test used to detect the production of acetoin, a metabolic by-product of glucose fermentation, by bacteria. It is commonly performed alongside the methyl red (MR) test as part of the IMViC series of tests used for the identification of Enterobacteriaceae and other Gram-negative bacteria.

The VP test is based on the ability of certain bacteria to convert glucose into a compound called 2,3-butanediol, which can be further oxidized to produce acetoin."

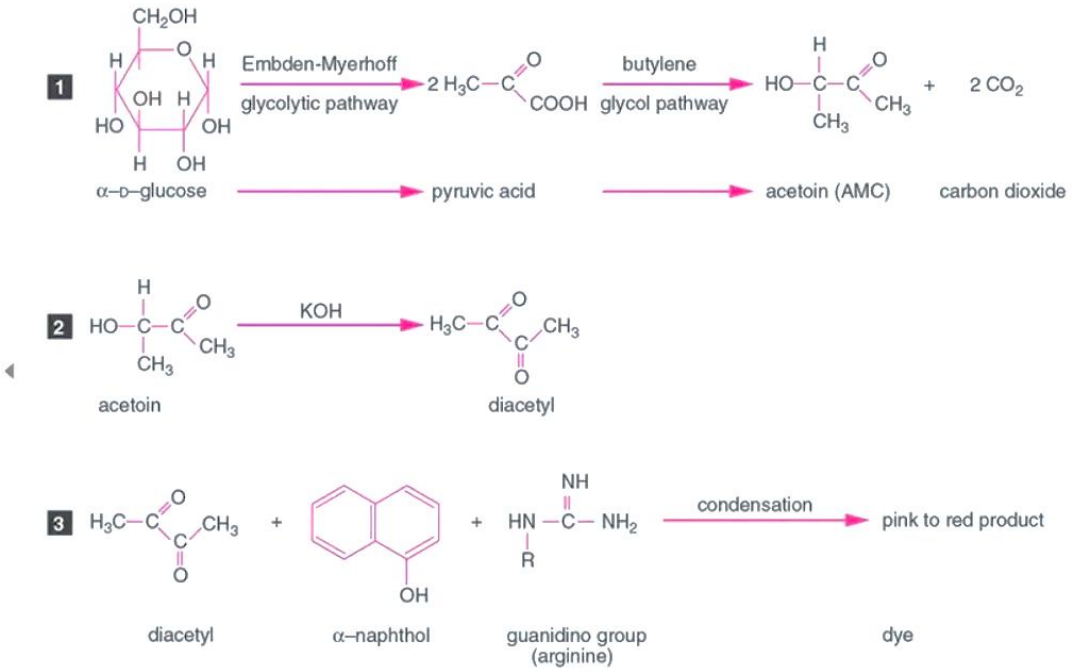


Fig. 4.4: VP Test

4.6.2.9 Methyl red Test

"The methyl red (MR) test is a biochemical test used to determine the ability of bacteria to produce and maintain stable acid end products from glucose fermentation. It is part of the IMViC series of tests commonly used for the identification of Enterobacteriaceae and other Gram-negative bacteria.

The MR test helps differentiate between bacteria that produce mixed acid fermentation products and those that produce a predominantly neutral fermentation pathway. Mixed acid fermentation produces a mixture of acids, including lactic acid, acetic acid, formic acid, and succinic acid, leading to a lowered pH of the culture medium."

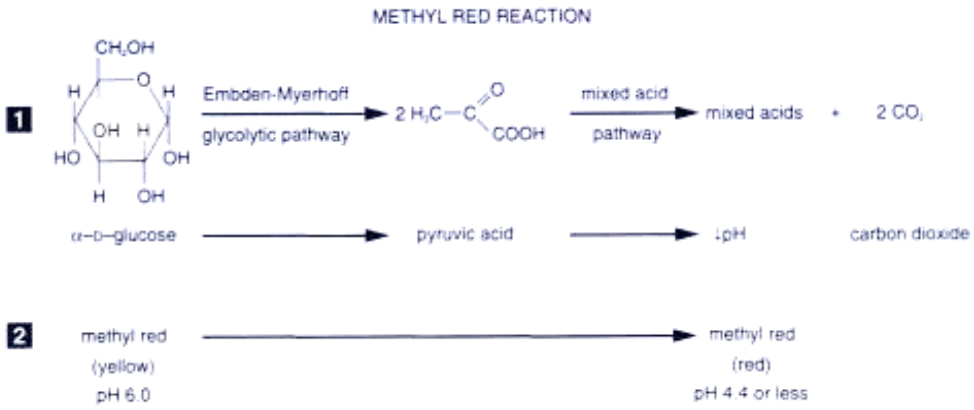


Fig. 4.5: Methyl red test

4.7 Antibiotic resistance profiling

"To determine the susceptibility of the isolates towards different antibiotics Kirby-Bauer disc diffusion assay was done.

The Kirby-Bauer disc diffusion assay, also known as the disk diffusion test, is a commonly used method in clinical microbiology to determine the susceptibility of bacteria to various antimicrobial agents. It provides a qualitative assessment of the effectiveness of different antibiotics against specific bacterial strains. Following steps were followed:

i. A bacterial culture is grown on a solid agar medium, such as Mueller-Hinton agar, to form a uniform lawn of bacterial growth.
ii. Small discs containing a defined amount of antibiotics are placed on the agar surface.
iii. The plate is then incubated under optimal conditions to allow the bacteria to grow.
iv. During incubation, the antibiotic diffuses out from the disc into the surrounding agar, creating a concentration gradient.
v. If the bacteria are susceptible to the antibiotic, growth inhibition zones, known as zones of inhibition, will form around the discs. The size of these zones is measured in millimeters and indicates the susceptibility of the bacteria to the antibiotic. Larger zones generally indicate greater susceptibility.
vi. The diameter of the inhibition zones is compared to standardized interpretive criteria provided by organizations like the Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST). These guidelines define breakpoints that categorize the

bacteria as susceptible, intermediate, or resistant to specific antibiotics.

By comparing the zone sizes to the interpretive criteria, healthcare professionals can determine the most appropriate antibiotics for treating bacterial infections. The results of the Kirby-Bauer test, along with other clinical and patient information, help guide antibiotic therapy decisions.

The antibiotic against which the susceptibility of the isolates was tested was selected based on recommendations given in CLSI 2019, antibiotics belonging to diverse classifications and generations were used to check the sensitivity of the isolates. Disc diffusion assays were done using antibiotic discs from HiMedia Laboratories (Mumbai, India). The sensitivity of the isolates against antibiotics (intermediate or susceptible) was deduced based on the information given in the manufacturer's manual. Results were interpreted based on CLSI guidelines.

We classified an isolate to be MDR if it exhibited resistance to antibiotics belonging to three or more classes."

Antibiotic	**Abb.**	**Conc. (µg/ml)**
Ampicillin	AMP	10
Methicillin	MET	30
Amoxiclav	AMC	30
Cephalothin	Cf	30
Vancomycin	VAN	30
Ceftriaxone	CTR	30
Cefoxitin	CX	30
Cefotaxime	CTX	30
Cefouroxime	CXM	30
Imipenem	I	10
Ciprofloxacin	CIP	10
Norfloxacin	NOR	10
Ofloxacin	OFX	5
Nalidixic acid	NAL	30
Levofloxacin	LE	5
Amikacin	AK	30
Streptomycin	S	10
Gentamicin	GEN	10

Erythromycin	ERY	15
Clindamycin	Cd	2
Doxycycline	DO	30
Tetracycline	TET	30
Chloramphenicol	C	30
Co-trimoxazole	COT	25

Table 4.1: List of antibiotics used and their concentrations

4.8 Molecular identification

"A single colony of individual bacterial culture was incubated into sterile Luria-Bertanni broth containing 10gL^{-1} tryptone, 5gL^{-1} sodium chloride and 5 gL^{-1} yeast extract at 37°C overnight. Genomic DNA was isolated from the overnight grown bacterial culture and with this isolated genomic DNA PCR was done using universal primers."

4.8.1 Isolation of genomic DNA from bacteria

"Genomic DNA from the overnight grown saturated bacterial culture was isolated using the following steps:

i. 1.5 ml of culture was centrifuged at 10,000 rpm for 10 minutes.
ii. The pellet was suspended in 500µL TE buffer of pH8.0 (containing 10mMTrisHCl of pH8.0 and 1mM EDTA of pH8.0).
iii. To the mixture lysozyme was added at 1mg.mL-1 concentration and incubated at 37°C for 1hr. To remove the RNA contamination RNaseH was added to the mixture at a concentration of 20µg mL^{-1}.
iv. 1% SDS was added to the mixture and incubated at 55°C for 15 minutes.
v. Equal volume of Tris saturated phenol was added to the mixture and mixed well by inverting the tube.
vi. Phase separation was performed by centrifuging the mixture at 10,000 rpm for 10 minutes.
vii. The aqueous phase was collected in a fresh centrifuge tube and a 1:1 mixture of Tris saturated phenol and chloroform was added at equal volume.
viii. The aqueous phase was separated and collected as above and an equal volume of chloroform was added to remove a trace amount of phenol present if any.
ix. The aqueous phase was again separated and collected in a fresh tube.
x. 0.3M sodium acetate and 2.5 volume chilled absolute ethanol was added to

facilitate the precipitation of the nucleic acids.

xi. After 1 hour incubation at -20°C centrifugation was performed at 15,000 rpm for 20 minutes.

xii. The pellet was washed with 70% ethanol twice and air dried.

xiii. Finally, the genomic DNA was dissolved in 30µL of 2.5mM Tris HCl (pH 8.0)."

4.8.2 Agarose gel electrophoresis for visualization of genomic DNA

"1% agarose gel was prepared and the isolated genomic DNA was visualized using the following protocol:

i. A stock solution of 50X TAE (Tris –Acetate-EDTA) buffer (pH 8.0) was prepared. It contains 242 g Tris base, 57.1mL glacial acetic acid and 100 mL of 500 mM EDTA (pH 8.0) per litre.

ii. It is diluted to 1X by mixing with an appropriate volume of distilled water.

iii. Agarose low EEO (SRL) was weighed according to 1% concentration.

iv. Agarose was dissolved in 1X TAE by heating.

v. After cooling down to room temperature 3 µL ethidium bromide was added per 100 mL of the gel.

vi. The gel was then poured over the clean gel casting tray and a comb was placed accordingly.

vii. The gel was allowed to solidify at room temperature.

viii. After solidification the comb was lifted and the gel was placed on a gel running tank filled with 1X TAE as a running buffer.

ix. The side of the well was placed towards the negative electrode.

x. 10µL DNA sample was mixed with 1X gel loading dye that contains bromophenol blue and xylene cyanol as indicator.

xi. The mixture was carefully loaded in a well using a micropipette.

xii. After completion the DNA was allowed to run through the gel powered by 100V current generated from a power pack.

xiii. As the forward dye bromophenol blue had crossed a definite distance, the current supply turned off and the band of the DNA was visualized by UV transilluminator."

4.8.3 Amplification of 16S rRNA gene using Polymerase chain reaction (PCR)

"16S rRNA gene fragment was amplified using the two 16S specific universal primers 27f and 1492r. The following calculations were used to formulate a master mix."

Ingredients	Volume
10 X PCR reaction Buffer (MgCl2 present)	2.5 µl
DNA	1 µl
dNTP (2.5mM)	2 µl
27f Primer	1 µl
1492r Primer	1 µl
Taq DNA polymerase (5U)	0.5 µl
Sterile water	X µl

Table 4.2: Master-mix composition used for PCR amplification

The temperature conditions required for PCR amplification are mentioned in the figure below.

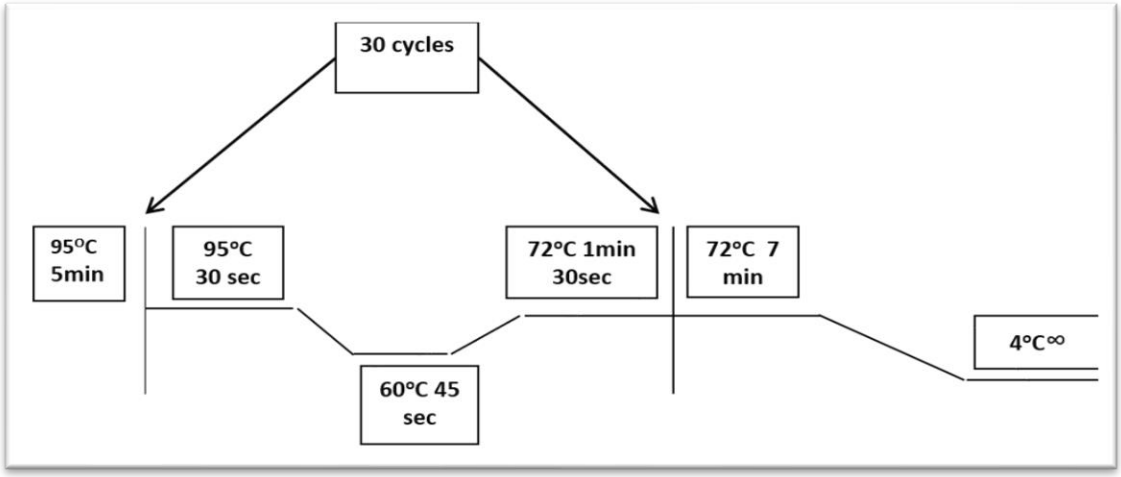


Fig.4.6: PCR amplification cycle

4.8.4 PCR product purification by agarose gel extraction

"The following steps were followed for PCR product purification by agarose gel extraction:

i. Cast 1 % agarose gel containing the PCR products.
ii. Observe under UV transilluminator. Cut the bands with a scalpel and place them in sterile eppendorfs.
iii. Calculate the weight and follow the QIAGEN Gel extraction kit procedure.
iv. Add 3 volumes of buffer QG to gel.
v. Incubate at 50°C for 10 minutes.
vi. Add 1 volume of Isopropanol and mix well.
vii. Place the sample in the spin column and centrifuge. Discard the flow through.
viii. Add 500 µl buffer QG and centrifuge.
ix. Add 750 µl buffer PE for washing and centrifuge.
x. Air dry for a minute.
xi. For elution, add 20 µl deionized water and centrifuge."

4.8.5 Sequencing of purified PCR products

i. "Purified PCR products are treated with 2 µl 125 mM EDTA (pH 8.0)
ii. Precipitated using 2 µl 3 M sodium acetate (pH 4.6) and 50 µl absolute ethanol for 20 min at room temperature
iii. DNA is recovered by centrifugation, washed with 70% alcohol, dried and re-suspended in 12µl Hi-Di formamide (Applied Biosystems, USA).
iv. After incubation in the dark for 20 minutes, denaturation at 96°C for 5 minutes.
v. Samples are kept at 4°C prior to the loading.
vi. Sequencing was performed in Applied Biosystems 3130XL Genetic Analyzer."

4.9 Phylogenetic analysis

"For constructing the phylogenetic tree, initially, two programs were used to generate the multiple sequence alignment for rapid validation of sequence scores:

- Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al. 2011) and
- Muscle (https://www.ebi.ac.uk/Tools/msa/muscle/) (Edgar 2004)).

Based on the consensus multiple sequence alignment, a phylogenetic tree was determined with 100 bootstrap replicates (Felsenstein1985) using the phylogenetic tree module of the CLC Genomics workbench. Here both neighbour joining-based methods (Saitou and Nei 1987) and maximum likelihood (Felsenstein, 1981) based methods

were tested to evaluate the tree generated and the best consensus tree was chosen for analysis and interpretation.

The tree file was visualised in FigTree to distinguish the different clades in specific colour codes."

4.10 Computational Analyses

The following targets were studied:

5TKW: *Klebsiella pneumoniae*

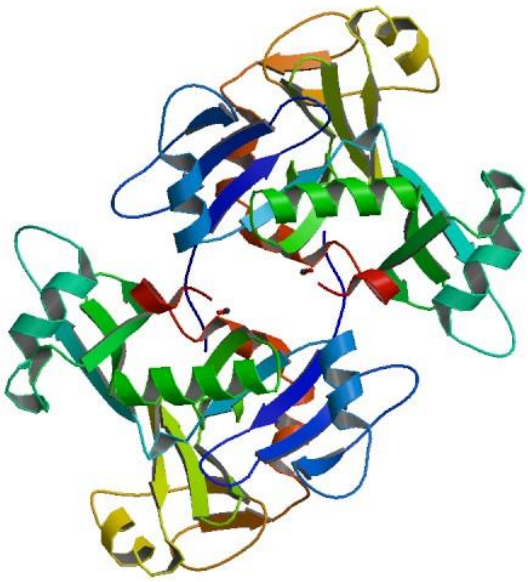


Fig. 4.7: 5TKW: 1.35 Angstrom Resolution Crystal Structure of a Pullulanase-specific Type II Secretion System Integral Cytoplasmic Membrane Protein GspL (N-terminal fragment; residues 1-237) from *Klebsiella pneumoniae*.

2XCQ: *Staphylococcus aureus*

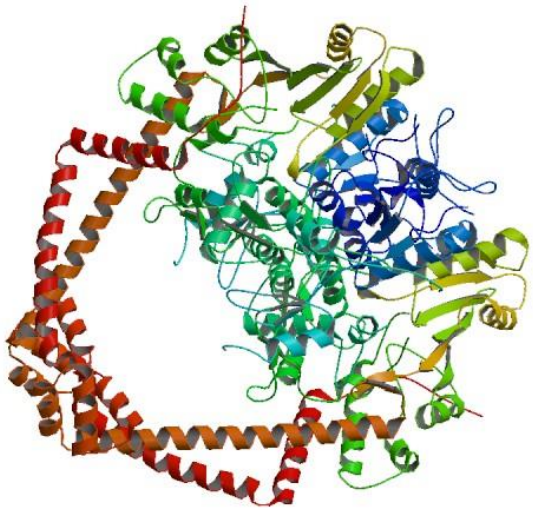


Fig. 4.8: "2XCQ: The 2.98A crystal structure of the catalytic core (B'A' region) of *Staphylococcus aureus* DNA Gyrase."

4E81: *E. coli*

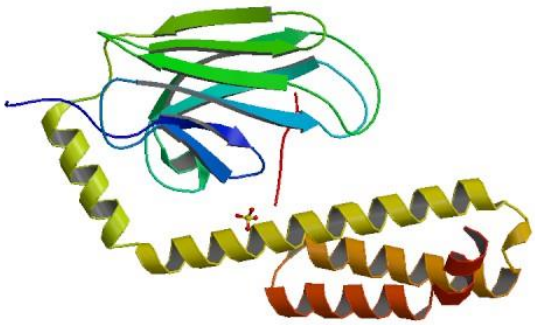


Fig. 4.9: Crystal structure of the substrate binding domain of *E. coli* DnaK in complex with a short apidaecin peptide

- "Crystal Structures of 5TKW, 4E8I and 2XCQ were downloaded from the Protein Data Bank [https://www.rcsb.org/]."
- "Details of the small molecules of *Christella* were studied from literature and noted. These were then searched for in the PubChem database [https:/ pubchem.ncbi.nlm.nih.gov/] and the list of compounds was curated."
- "The compounds were downloaded in .sdf format and converted to .pdb using the online SMILES translator tool [https:// cactus.nci.nih.gov/translate/]; following this they were tested for their druggability and bioactivity using the MOLINSPIRATION server [http://www.molinspiration.com/] (Ganguli *et. al*, 2011; Salma *et. al*, 2017)."
- "The PatchDock server [https://bioinfo3d.cs.tau.ac.il/PatchDock/] was used to analyze the propensity of complex formation through molecular docking and the atomic contact energies were noted (Duhovny *et. al*, 2005)."
- "Protein-ligand complexes were then analyzed for their interactions using Ligplot. [https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/] (Wallace *et. al*, 1995)."

List of active compounds from *Christella* for docking studies:

- Beta sitosterol
- Hexacosyl hexadecanoate
- Kaempferol
- Matteucinol"

5. RESULTS

5. Results

5.1 Evaluation of physicochemical parameters of the effluents

"The physico-chemical parameters of the effluents were identified with the purpose of observing driving factors behind widespread antibiotic resistance and horizontal gene transfer.

Heavy metals such as Cd, Pb, Hg were found to be higher than the permissible limits in both effluents (Fig. 1a). Total Phosphorus was higher than the permissible limits. COD was also found to be higher than the permissible limits (Fig. 1b)."

Parameters		**Results**
1.	TOC (mg./l)	120.0
2.	Oil & Grease (mg./l)	3.50
3.	TS (mg./l)	1650.0
4.	TSS (mg./l)	35.0
5.	Total Dissolved Solids (mg./l)	1250.0
6.	TVS (mg./l)	245.0
7.	TFS (mg./l)	60.0
8.	NH_3 (mg./l)	3.80
9.	Total Kjeldhal Nitrogen (mg./l)	8.50
10.	Nitrate (mg./l)	2.80
11.	Total Phosphorus (mg./l)	6.80
12.	Arsenic (mg./l)	<0.01
13.	Cadmium (mg./l)	1.20
14.	Chromium (mg./l)	<0.02
15.	Lead (mg./l)	1.60
16.	Mercury (mg./l)	1.20

Table 5.1: Physicochemical parameters of LS sample

Parameters		**Results**
1.	COD (mg./kg.)	310.0
2.	TOC (mg./kg.)	105.0
3.	TS (gm/gm)	0.78
4.	TVS (gm/gm)	0.32
5.	NH_3 (mg./kg.)	6.50
6.	Total Kjeldhal Nitrogen (mg./kg.)	16.50
7.	Nitrate (mg./kg.)	6.80
8.	Total Phosphorus (mg./kg.)	12.80
9.	Arsenic (mg./kg.)	0.80
10.	Cadmium (mg./kg.)	2.50
11.	Chromium (mg./kg.)	<0.5
12.	Lead (mg./kg.)	6.50
13.	Mercury (mg./kg.)	1.20

Table 5.2: Physicochemical parameters of SS sample

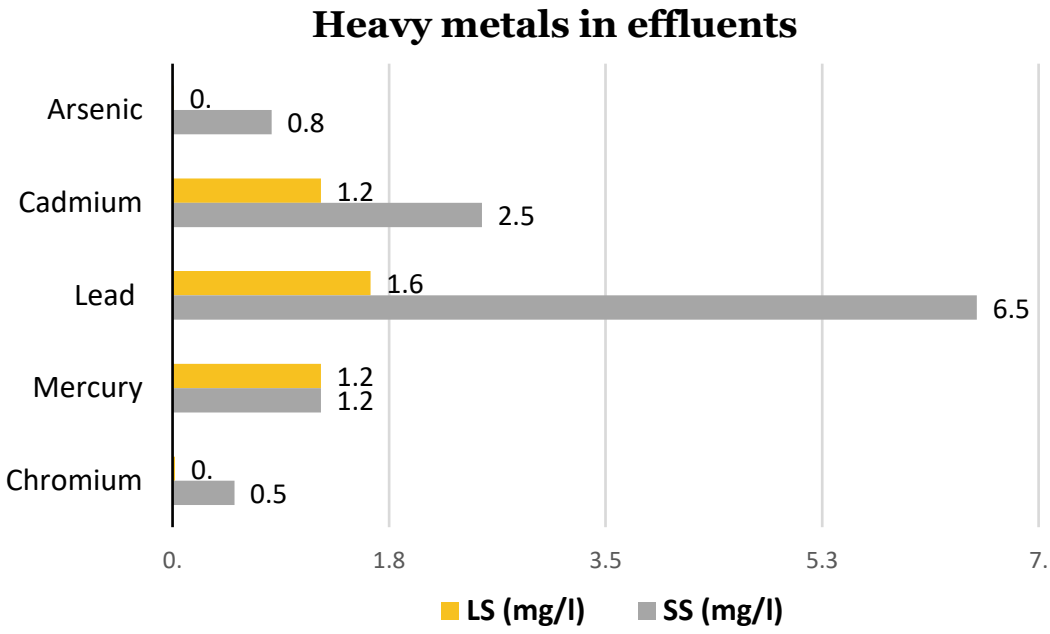


Fig. 5.1: "Heavy metal concentrations in samples (Graphical representation of the estimation of heavy metal estimation in LS and SS sample sets with parameters on y-axis and concentrations (mg/l) on x-axis)"

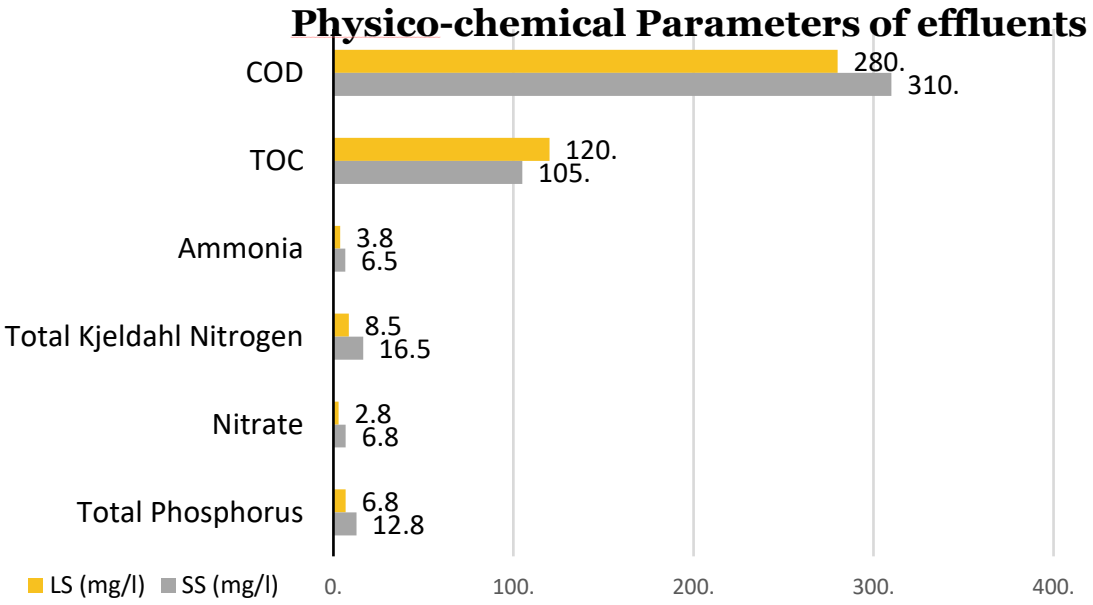


Fig. 5.2: "Physico-chemical parameters of samples (Graphical representation of concentration of various analytes for LS and SS sample sets with parameters on y-axis and concentrations (mg/l) on x-axis)."

5.2 Metagenomics profiling

Data set LS

The data set LS contains 15,372,973 reads with an average length of 301 bps as shown in Fig. Of the sequences that passed QC, 28,095 sequences (93%) contain ribosomal RNA genes, 1,104 sequences (3.65%) contain predicted proteins with known functions, and 1,069 sequences (3.53%) contain predicted proteins with unknown function. The community study revealed an abundance of 19.3 % for the members of Bacteroidetes. At the genus level, Prevotella was the most dominant microbial member with abundance of 17.47%

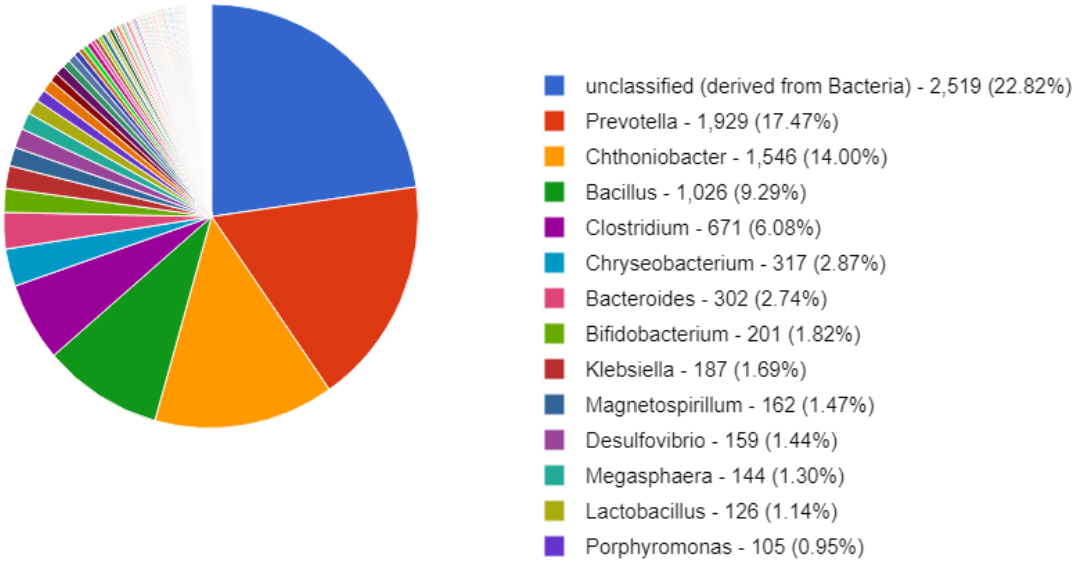


Fig. 5.3: Pie chart representing genus diversity of the effluent metagenome LS

Data set SS

"The data set SS contains 16,071,594 reads with an average length of 301 bps represented by Fig. Of the sequences that passed QC, 12,171 sequences (59%) contain ribosomal RNA genes, 1,885 sequences (9.21%) contain predicted proteins with known functions, and 6,403 sequences (31.30%) contain predicted proteins with unknown function. The community study revealed an abundance of 19.7 % for the members of Euryarchaeota. Methanosaeta was the dominant genus with abundance of 16.47%."

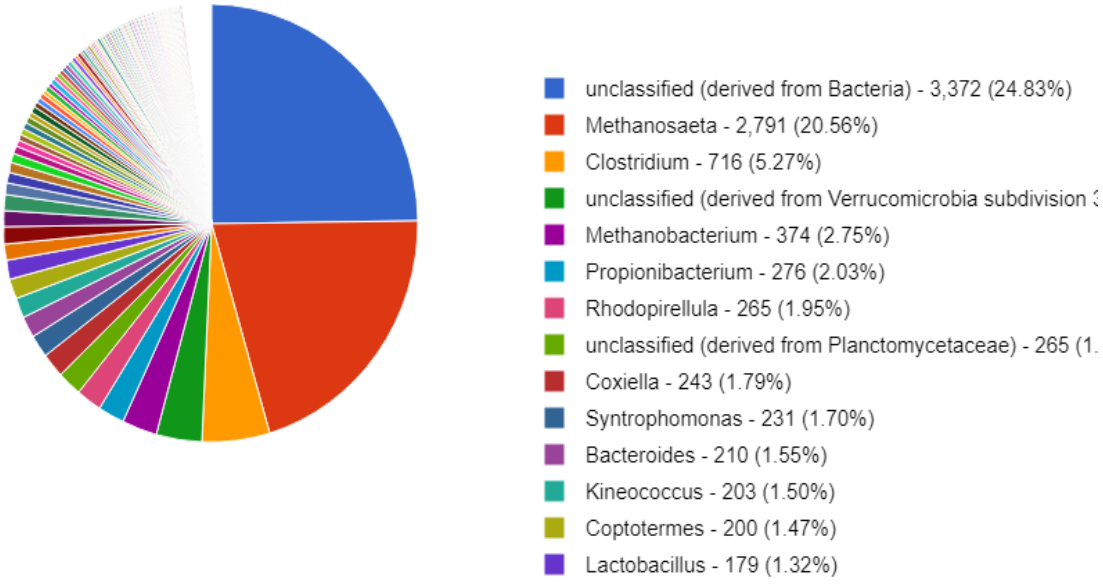


Fig. 5.4: Pie chart representing genus diversity of the effluent metagenome SS

Comparative genus diversity

The comparative genus diversity LS and SS sample sets is represented by Fig. 5. The common genera among the two datasets were:

- *Prevotella*
- *Bacillus*
- *Clostridium*
- *Bacteroides*
- *Bifidobacterium*
- *Lactobacillus*
- *Porphyromonas*
- *Propionibacterium*
- *Prevotella*
- *Bacillus*
- *Clostridium*
- *Bacteroides*
- *Bifidobacterium*
- *Lactobacillus*
- *Porphyromonas*
- *Propionibacterium*

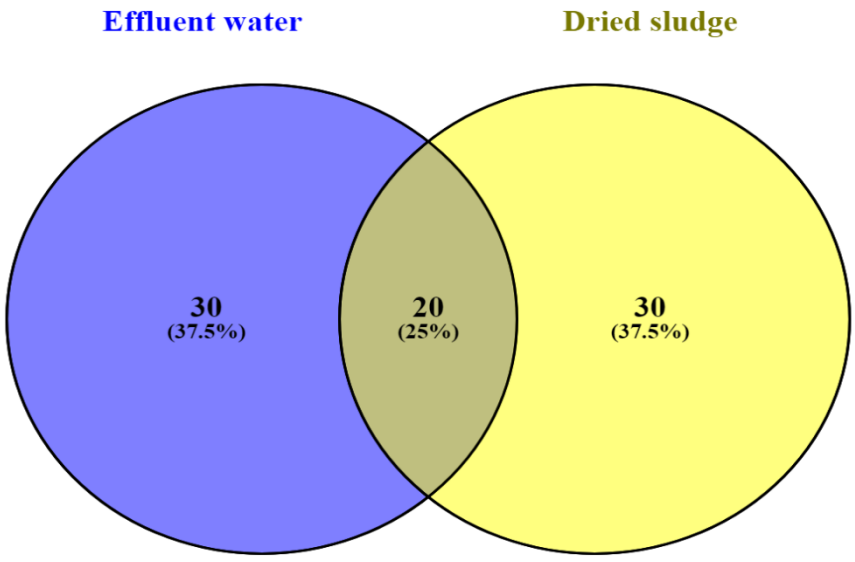


Fig. 5.5: "Venn diagram representing the common genus among the LS and SS samples."

Pathogenicity of total bacterial load

The pathogenic potential of the bacterial load from both the effluents was determined using Microbiome Analyst server and it was found that it can cause diseases/disorders such as bacterial vaginosis, irritable bowel syndrome (IBS), COPD, infectious colitis etc.

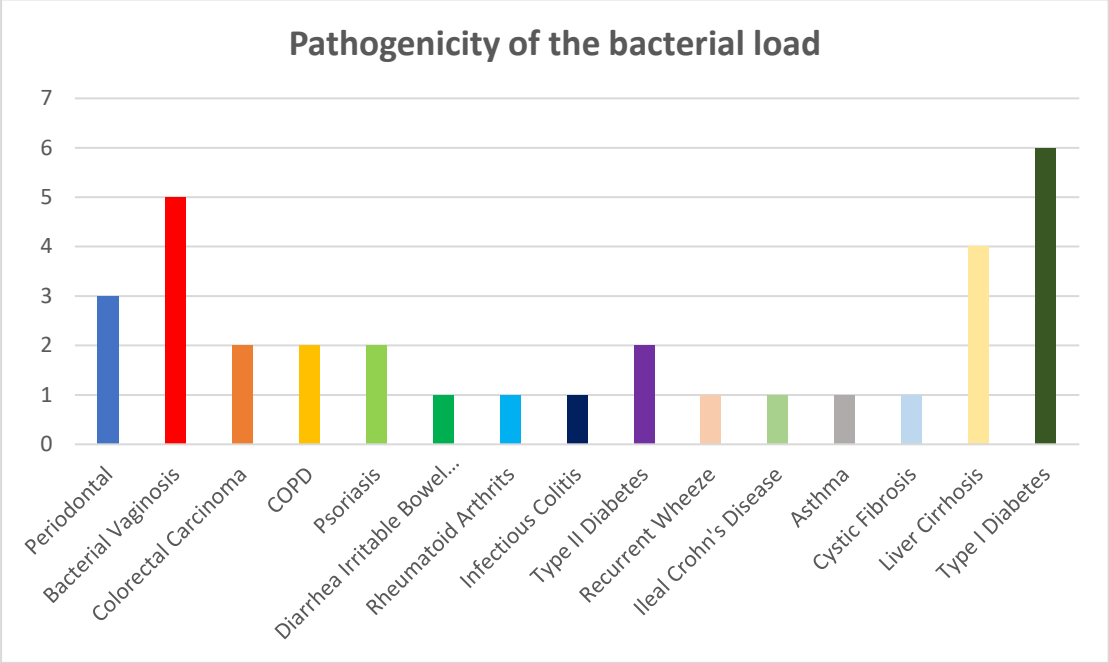


Fig. 5.6: Total pathogenic load of both effluents

5.3 Taxonomic diversity determination

For LS dataset, α-diversity is 27 species. The value of Shannon index is 2.56.

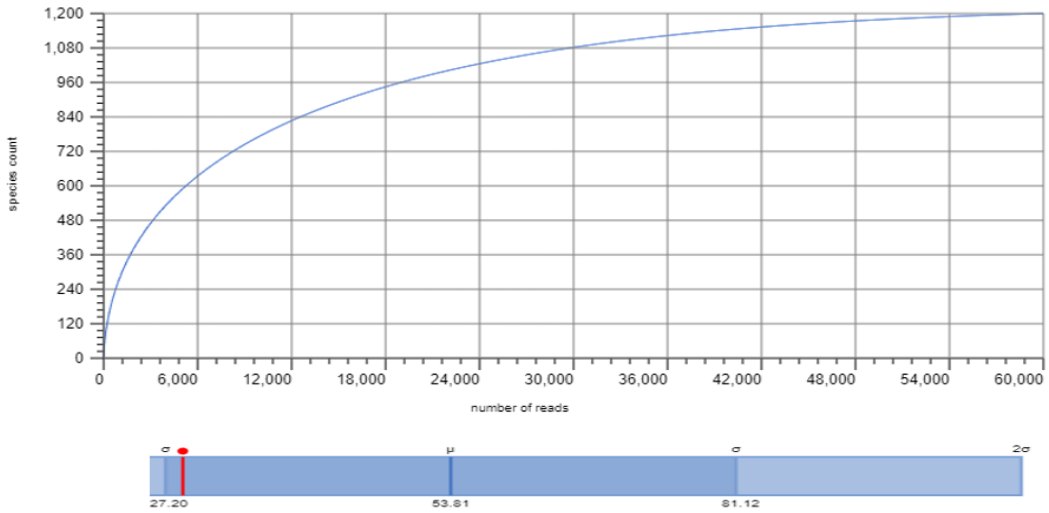


Fig 5.7: Rarefaction curve representing species count (y-axis) against number of reads (x-axis) for LS dataset

For SS dataset, α-diversity is 77 species. The value of Shannon index is 2.75.

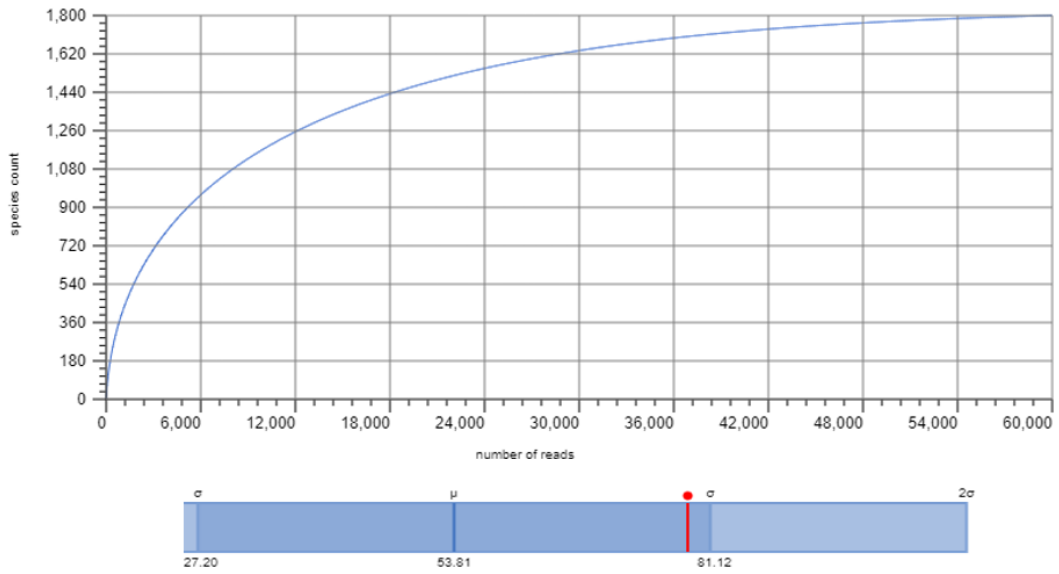


Fig. 5.8: Rarefaction curve representing species count (y-axis) against number of reads (x-axis) for SS dataset

5.4 Taxon set enrichment analysis

"From the metagenomics-based analysis, generation of a predicted pathogenic load network graph is possible which considers taxon enrichment and enables to correlate the data with genus abundance profiles."

	Query	Genera Enriched
1	Clostridiales;	
2	Lactobacillales;	
3	Ruminococcus	Ruminococcus
4	Lactobacillales;f__;g__	
5	Enterobacteriaceae;Other	
6	Enterococcaceae;Other	
7	Fusobacterium	Fusobacterium
8	Actinomyces	Actinomyces
9	Pseudomonadaceae;Other	
10	Bacteroides	Bacteroides
11	Veillonella	Veillonella
12	Enterobacteriaceae;g__	
13	Streptococcus	Streptococcus
14	Bifidobacterium	Bifidobacterium
15	Collinsella	Collinsella
16	Bulleidia	Bulleidia
17	Prevotella	Prevotella
18	Lactobacillus	Lactobacillus
19	Coprococcus	Coprococcus
20	Dorea	Dorea
21	Eggerthella	Eggerthella
22	Coriobacteriaceae;g__	
23	Clostridiales;f__;g__	
24	Parabacteroides	Parabacteroides
25	Lachnospiraceae;g__	
26	Faecalibacterium	Faecalibacterium
27	Blautia	Blautia
28	Pseudoramibacter_Eubacterium	
29	Lachnospiraceae;Other	
30	Streptococcaceae;Other	
31	Rikenellaceae;g__	
32	Firmicutes;	
33	Streptophyta;f__;g__	
34	Gemellaceae;g__	
35	Clostridiaceae;g__	
36	Rothia	Rothia
37	Porphyromonas	Porphyromonas
38	Capnocytophaga	Capnocytophaga
39	Leptotrichia	Leptotrichia
40	Haemophilus	Haemophilus
41	Treponema	Treponema
42	Methanobrevibacter	
43	Peptostreptococcus	Peptostreptococcus
44	Sutterella	Sutterella
45	Acinetobacter	Acinetobacter
46	Pseudomonas	Pseudomonas
47	Mycoplasma	Mycoplasma
48	Akkermansia	Akkermansia

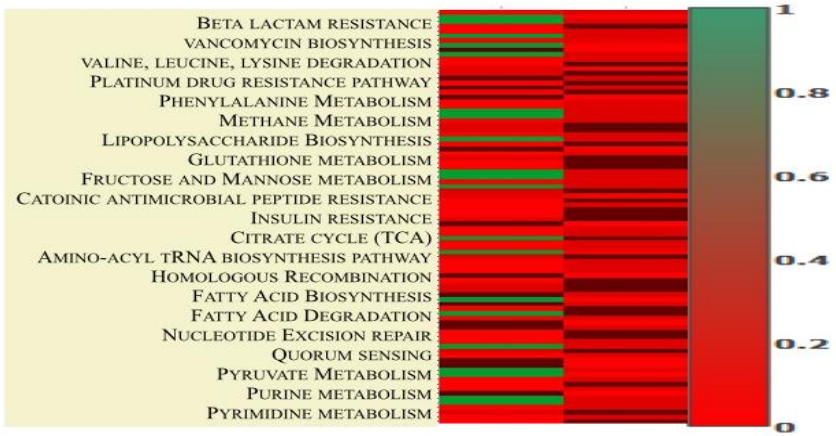


Fig. 5.9: "Enriched genera and corresponding enriched pathways in the wastewater niche."

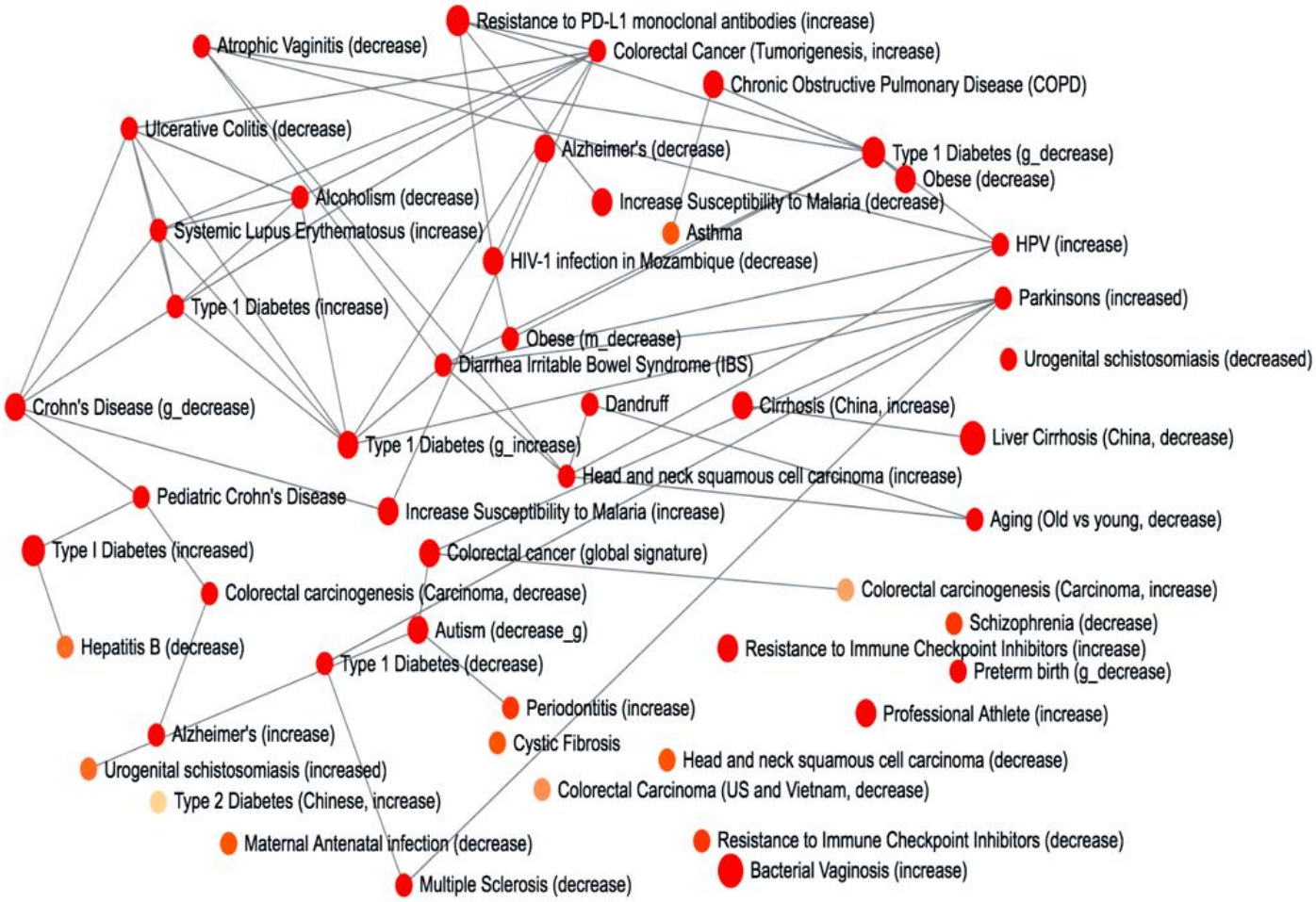


Fig.5.10: Pathogenic load analyses

5.5 Pathway analyses

- The microorganisms present in rural sample (LS) have higher number of multidrug resistance genes, multiple antibiotic resistance, and stress resistance proteins than that in urban sample (SS).
- The microorganisms in LS sample are rich in penicillin-binding proteins.
- The microorganisms in SS sample have higher number of Biofilm regulatory and synthesis genes.
- Metal resistance genes such as copper and mercuric resistance genes are higher in microorganisms of LS sample set.

S. No.	**Gene**	**Pathway ID**	**No. of DEG**	
			LS	**SS**
1.	Multidrug resistance protein, MATE family	K03327	139	73
2.	Multidrug resistance protein A	K03543	135	0
3.	Multiple antibiotic resistance protein	K05595	118	68
4.	Multiple antibiotic resistance protein MarB	K13630	7	0
5.	Multiple stress resistance protein BhsA	K12151	7	0
6.	Penicillin amidase	K01434	7	50
7.	Penicillin-binding protein 1A	K05366	221	67
8.	Penicillin-binding protein 1B	K05365	27	0
9.	Penicillin-binding protein 1C	K05367	20	0
10.	Penicillin-binding protein 2	K05515	138	102
11.	Copper resistance protein B	K07233	64	0
12.	Mercuric resistance operon regulatory protein	K08365	36	0

13.	Biofilm PGA synthesis lipoprotein PgaB	K11931	7	15
14.	Biofilm PGA synthesis N-glycosyltransferase PgaC	K11936	14	15
15.	Biofilm PGA synthesis protein PgaD	K11937	7	15
16.	Biofilm regulator BssS	K12148	7	0

Table 5.3: Antibiotic and metal resistance genes in both LS and SS samples

5.6 Microbiological characterization

5.6.1 Morphological characterization

After serial dilution, the effluent samples were plated onto nutrient agar plates and kept for overnight incubation (37 °C). Isolated colonies in higher dilution were observed on each type of plates. These colonies were then sub-cultured in nutrient agar plates for pure colony isolation.

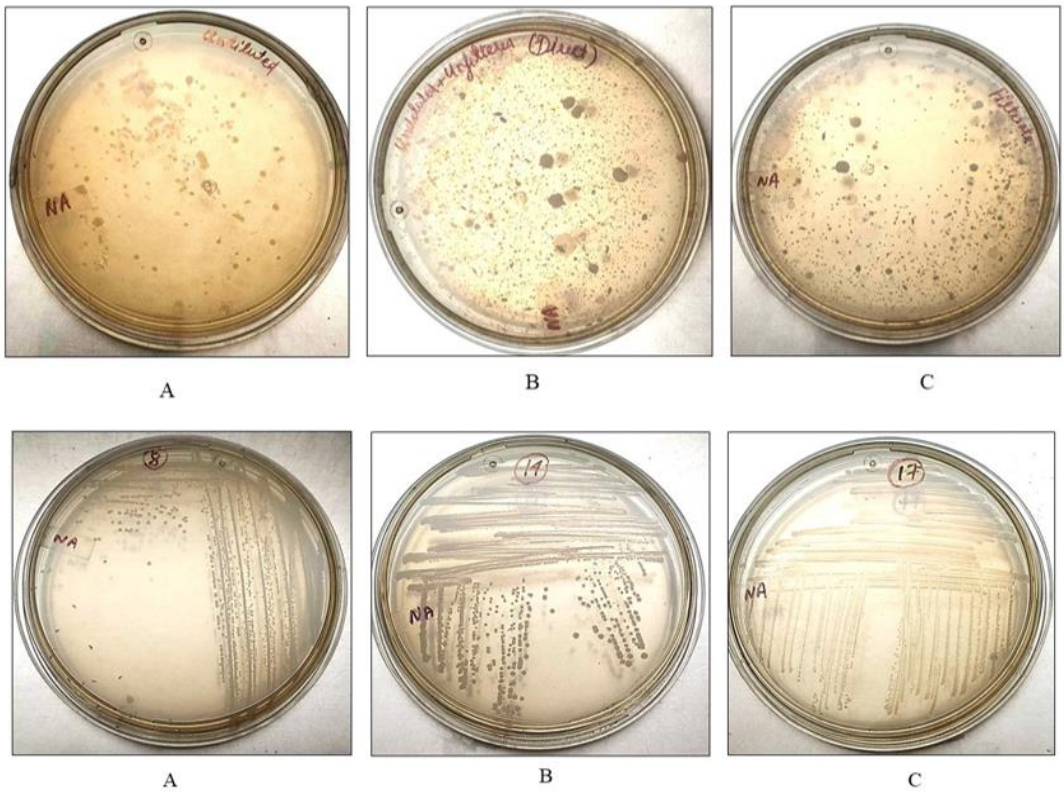
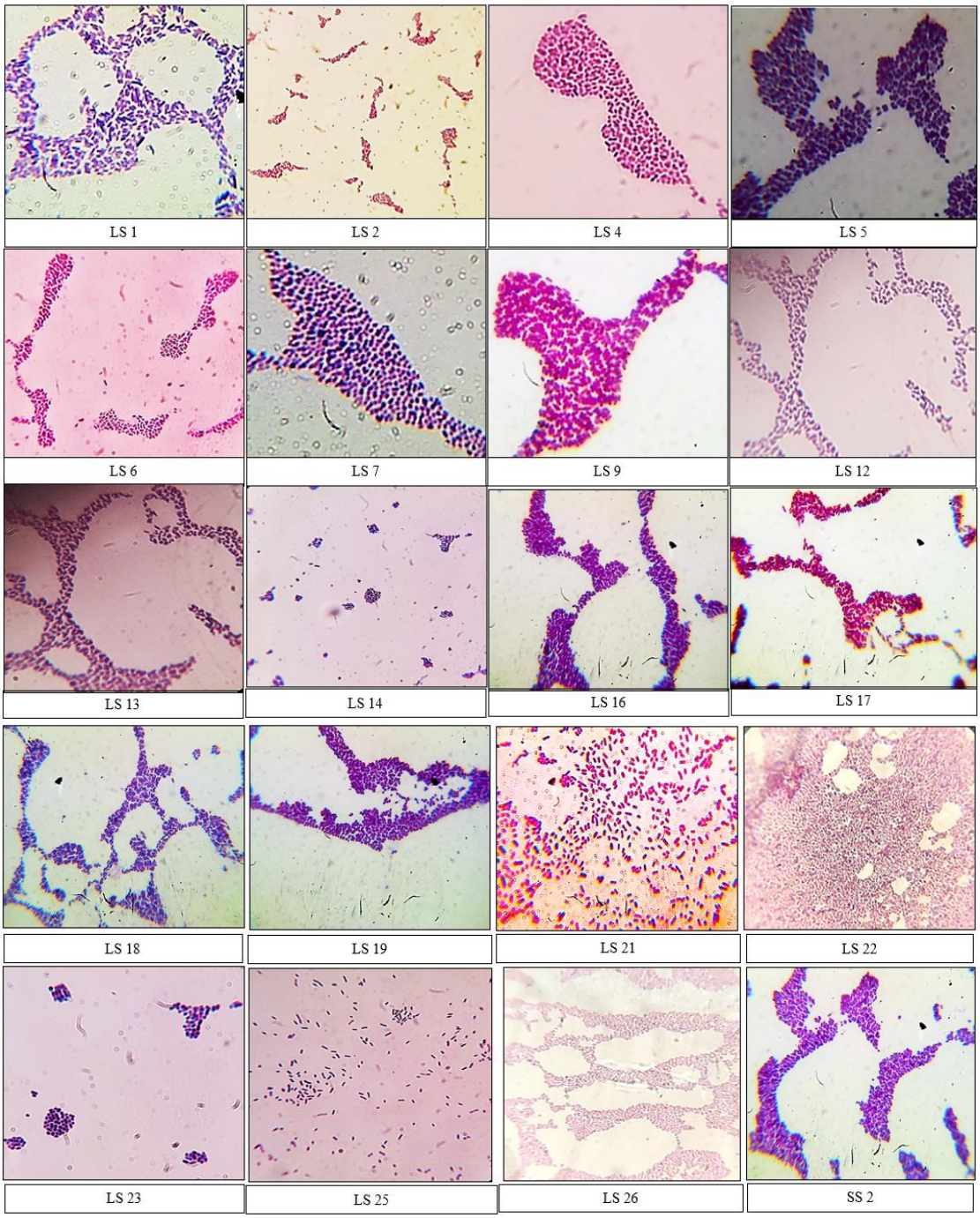


Fig. 5.11: Morphological characterization of bacteria and pure colony isolation

Gram character determination

"The total number of isolated bacteria for LS and SS datasets was 19 and 23 respectively. The study was focused on multidrug resistant strains due to it was narrowed down to 14 isolates with 10 isolates from LS dataset and 4 isolates from SS dataset. Among the selected isolated bacteria, 8 were Gram negative rods (LS 2, LS 4, LS 6, LS 9, LS 17, LS 21, SS 7 and SS 19) and 6 were Gram-positive rod-shaped bacteria (LS 12, LS 14, LS 21, LS 23, LS 25 and SS 20)."



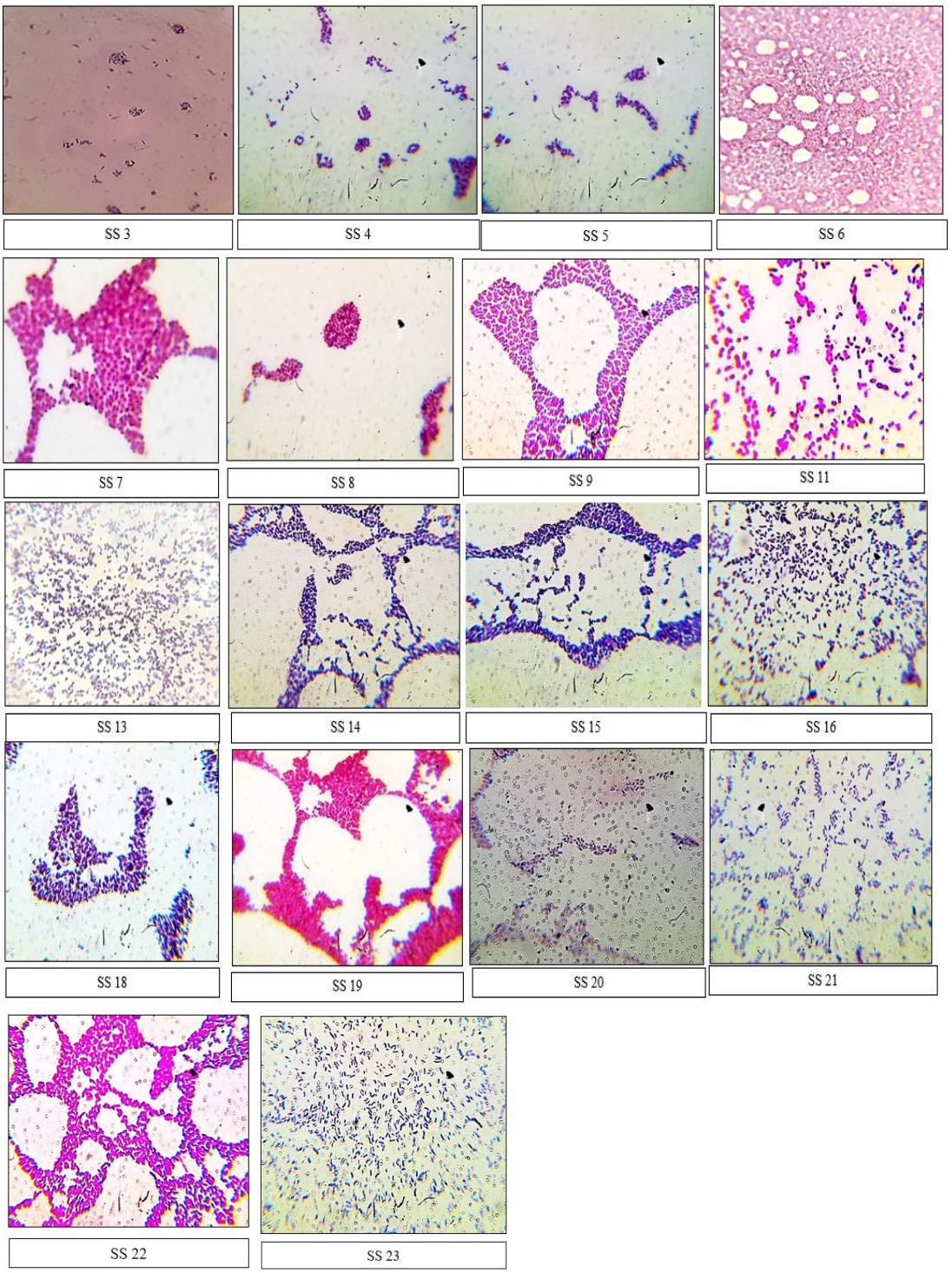


Fig. 5.12: Gram staining images of isolates as observed under 100x Magnification of objective lens of Bright Field Light Microscope

LS	Gram Character	Cellular Morphology
1	-ve	Spherical in small clusters
2	+ve	Spherical in dense clusters
4	+ve	Spherical in clusters
5	+ve	Small Rods singly and small clusters.
6	-ve	Small Rods small clusters
7	+ve	Long Rods in clustered pattern
9	-ve	Small Rods in cluster
12	+ve	Small rods and in clustered pattern.
13	+ve	Spherical and highly clustered
14	+ve	Spherical in clusters
16	+ve	Spherical, singly present
17	+ve	Small rods in clusters
18	+ve	Long Rods in clustered pattern
19	+ve	Long Rods singly present
21	+ve	Spherical in clusters
22	-ve	Long Rods in clusters
25	+ve	Small Rods singly and in small clusters
26	-ve	Spherical in clusters
27	-ve	Long rods in small clusters

5.4: Gram Character and Cellular Morphology of Bacteria Isolated from LS sample

SS	Gram Character	Colony Morphology
2	-ve	Small rods in clustered pattern.
3	+ve	Small rods singly present
4	+ve	Small rods in small clusters.
5	+ve	Small Rods singly present.
6	+ve	Small Rods singly present.
7	+ve	Small rods in small clusters.
8	+ve	Small rods singly and in clusters.
9	+ve	Spherical in dense clustered pattern.
11	+ve	Small Rods singly present.
12	+ve	Small Rods singly present.
13	+ve	Small Rods in clusters.
14	+ve	Small rods singly present.
15	+ve	Small rods singly present.
16	-ve	Small rods in clustered pattern
17	+ve	Small rods singly present.
18	-ve	Small rods in clustered pattern
19	+ve	Spherical in small clusters
20	+ve	Small rods singly present.
21	+ve	Small rods in scattered arrangement.
22	+ve	Long rods in clustered pattern.
23	+ve	Long rods singly present

Table 5.5: Gram Character and Cellular Morphology of Bacteria Isolated from SS sample

5.6.2 Biochemical characterization

The isolates were subjected to various biochemical tests in order to determine the genus and species.

L.S	Catalase Test	Indole Test	Starch Hydrolysis Test	Citrate Utilization Test	VP Test	Methyl Red Test	Oxidase Test	Nitrate Reduction Test	Urease Production Test
1	+	+	+	-	+	+	-	+	-
2	+	-	-	+	+	+	+	+	-
4	+	-	+	-	-	+	+	+	-
5	+	-	+	+	+	-	+	+	-
6	-	-	+	+	-	-	+	+	+
7	-	+	+	+	+	+	+	+	-
9	+	-	+	-	+	+	-	+	-
12	+	-	+	+	+	-	+	+	-
13	+	-	+	+	+	-	+	+	+
14	+	-	+	+	+	-	+	+	-
16	+	+	+	+	+	+	+	+	-
17	+	-	+	+	+	+	+	+	-
18	+	-	+	+	+	-	+	+	-
19	+	-	-	-	+	+	+	+	-
21	+	+	+	-	+	+	+	+	-
22	+	-	+	-	+	+	+	+	-

25	+	-	+	-	+	+	+	+	-
26	+	-	+	-	+	+	+	+	-
27	+	-	-	-	+	+	-	+	-

Table 5.6: LS dataset biochemical profiling

S.S	Catalase Test	Indole Test	Starch Hydrolysis Test	Citrate Utilization Test	VP Test	Methyl Red Test	Oxidase Test	Nitrate Reduction	Urease Production
2	+	-	+	+	+	-	+	-	-
3	-	-	+	+	+	-	+	-	-
4	+	-	+	+	-	-	+	-	-
5	+	-	+	+	+	-	-	+	-
6	-	-	-	+	-	-	+	-	-
7	+	+	+	+	+	-	+	-	-
8	+	-	-	-	-	-	+	+	-
9	+	-	+	+	+	-	-	-	-
11	-	+	+	-	+	-	-	-	-
12	+	-	+	+	-	-	-	-	-
13	+	-	+	-	-	-	-	-	-

14	+	-	+	+	-	-	+	-	-
15	+	-	+	-	-	+	+	-	-
16	-	-	+	-	+	+	+	-	-
17	+	-	+	-	-	-	+	+	-
18	+	-	+	+	+	-	-	+	-
19	+	-	+	-	-	-	-	-	-
20	+	-	-	-	+	-	-	-	-
21	+	-	+	+	-	-	+	+	-
22	+	-	-	-	-	-	+	+	-
23	-	-	-	-	-	-	+	-	-

Table 5.7: SS dataset biochemical profilin

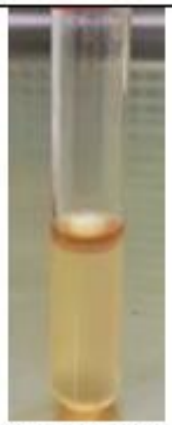
LS	Glucose		Lactose		Sucrose		SS	Glucose		Lactose		Sucrose	
	Gas	Color	Gas	Color	Gas	Color		Gas	Color	Gas	Color	Gas	Color
LS1	+	Yellow	-	Yellow	-	Red	SS2	-	Yellow	+	Yellow	Red	-
LS2	+	Yellow	+	Yellow	-	Yellow	SS3	-	Yellow	-	Red	Yellow	-
LS4	-	Yellow	+	Red	+	Red	SS4	-	Yellow	-	Red	Red	-
LS5	+	Yellow	-	Yellow	-	Yellow	SS5	-	Yellow	-	Red	Red	-

LS6	+	Yellow	-	Red	-	Yellow	SS6	-	Yellow	-	Red	Red	-
LS7	+	Yellow	-	Red	+	Yellow	SS7	-	Yellow	-	Red	Yellow	-
LS9	-	Yellow	-	Red	-	Red	SS8	-	Yellow	-	Red	Red	-
LS12	+	Yellow	-	Yellow	-	Yellow	SS9	+	Yellow	-	Red	Red	-
LS13	+	Yellow	+	Yellow	+	Yellow	SS11	+	Yellow	-	Red	Red	-
LS14	-	Yellow	+	Red	-	Yellow	SS12	-	Red	-	Red	Yellow	-
LS16	+	Yellow	-	Yellow	-	Yellow	SS13	-	Yellow	-	Red	Yellow	-
LS17	-	Yellow	-	Red	+	Red	SS14	-	Red	-	Red	Red	-
LS18	+	Yellow	-	Yellow	-	Yellow	SS15	-	Yellow	-	Red	Red	-
LS19	-	Yellow	-	Red	-	Red	SS16	+	yellow	-	Red	Red	-
LS21	-	Yellow	-	Yellow	-	Red	SS17	-	yellow	-	Red	Yellow	-
LS22	-	Yellow	-	Red	-	Red	SS18	-	Red	-	Red	Red	-
LS25	-	Yellow	-	Yellow	-	Red	SS19	-	Red	-	Red	Red	-
LS26	-	Yellow	-	Red	-	Red	SS20	-	Red	-	Red	Yellow	-
							SS21	-	Red	-	Red	Red	-
							SS22	-	Red	-	Red	Red	-
							SS23	-	Red	-	red	Red	-

Table 5.8: Carbohydrate Fermentation Test of both LS and SS datasets

S.No.	Biochemical Test	Negative result	Positive result
1.	Urease Test	 No change in colour of the media.	 Change in colour of the media to pink.
2.	Catalase Test	 No effervescence formation	 Effervescence production
3.	Nitrate reduction Test	 No colour development at first.	 Development of deep red colour

4.	Starch hydrolysis Test	 No zone of clearance	 Zone of clearance indicative of starch hydrolysis
5.	Oxidase Test	 No colour development on addition of oxidase reagent	 Development of intense deep blue colour
6.	Citrate utilization test	 No change in colour of the pH indicator	 Change in colour of the pH indicator bromothymol blue

7.	Indole test	 Clear upper alcoholic layer	 Reddish ring in upper alcoholic layer
8.	Methyl red test	 No change in colour of the acid indicator	 Change in colour of methyl red indicator from yellow to red
9.	Voges Proskauer Test	 No change in colour	 Development of copperish red colour

10.	Carbohydrate fermentation test	 No acid or gas production	 Gas production	 Acid production	 Gas and Acid production

Table. 5.9: Biochemical characterization results

5.7 Antibiotic sensitivity profiling

"The antibiotic resistance was determined in terms of zone of inhibition (cm) data and it was found that isolates obtained from Purulia (rural area) was found to be much higher than that in Kolkata (urban area).

This study identifies 83.9% of the isolates to exhibit resistance to beta lactams with 100% resistance to Ampicillin. Conte et al., 2017 reported higher probability of occurrence of ESBL-producing K. pneumonia and E. coli isolates in hospital effluent, Waste Water Treatment Plants (WWTP) and river samples, respectively whereas, hospital effluent, sanitary effluent, outflow sewage and surface water samples were richer in quinolone resistant isolates. In this study, high macrolide resistance among the isolates (85.7%) which clearly points to the diverse antibiotic resistance potential of the effluents. The resistance to fluoroquinolones was 44.2 %, 50% for glycopeptides and cephalosporins, 35.7 % for carbapenems and sulfonamides, 28.5 % for tetracycline, 23.8% for aminoglycosides. All the isolates were however, found to be susceptible to chloramphenicol and streptomycin which may be because these antibiotics are prescribed very sparsely by healthcare professionals and medical practitioners in the vicinity of the collection spots."

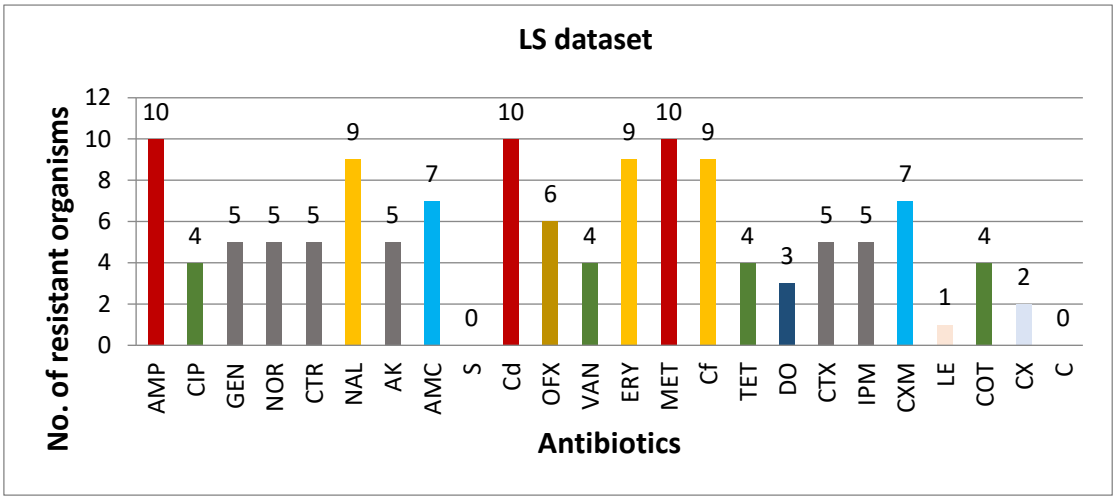


Fig. 5.13: "Graphical representation of the number of resistant isolates in LS sample set against various antibiotics; the number of resistant isolates is represented on y-axis and the antibiotics on x-axis."

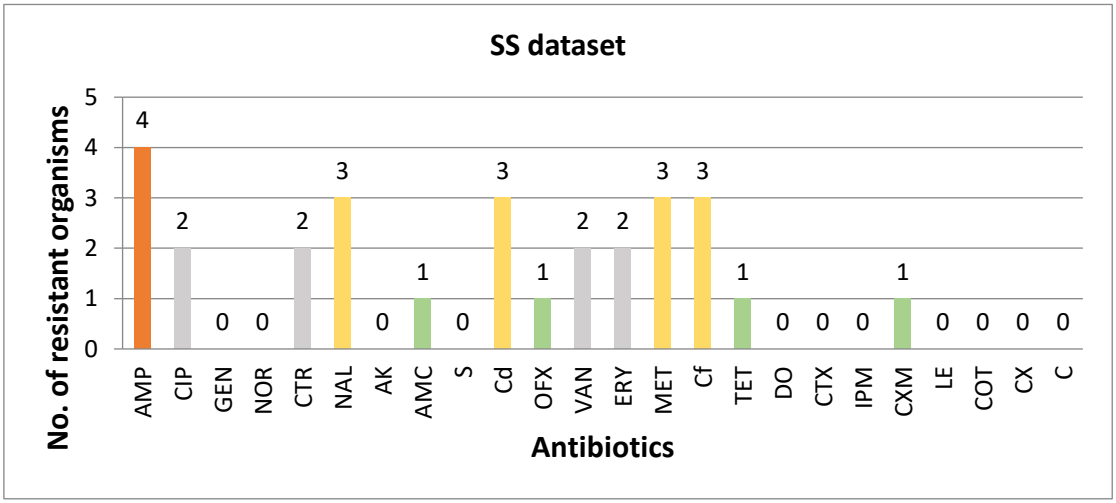
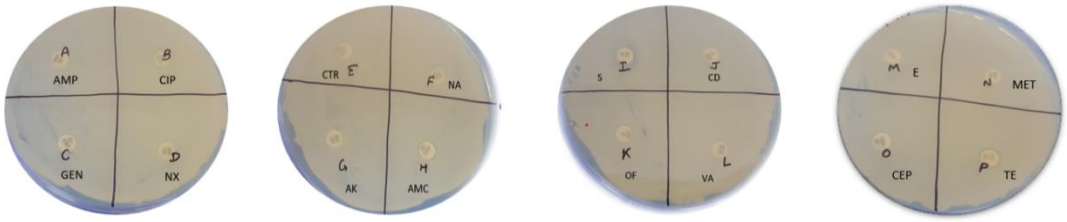
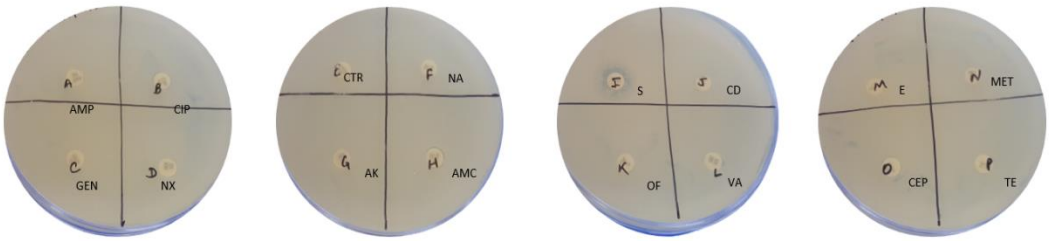


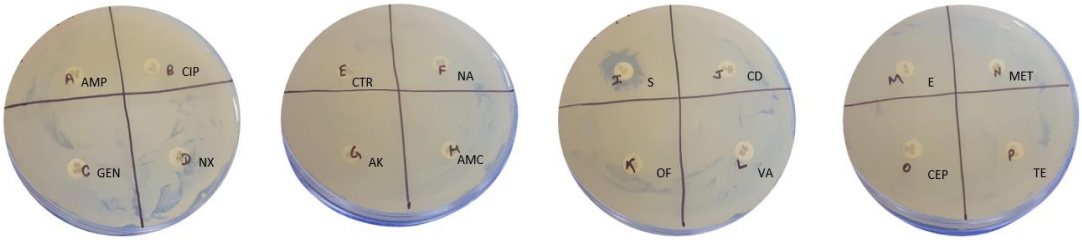
Fig. 5.14: "Graphical representation of the number of resistant isolates in SS sample set against various antibiotics; the number of resistant isolates is represented on y-axis and the antibiotics on x-axis."



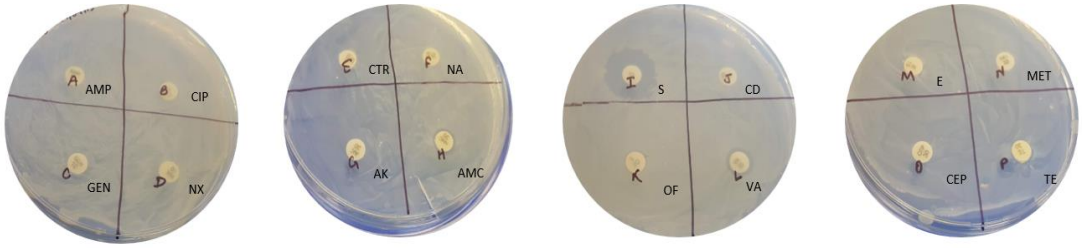
Isolate: LS 2



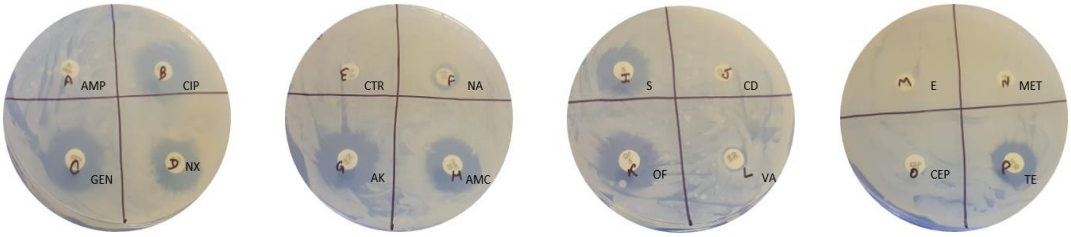
Isolate: LS 4



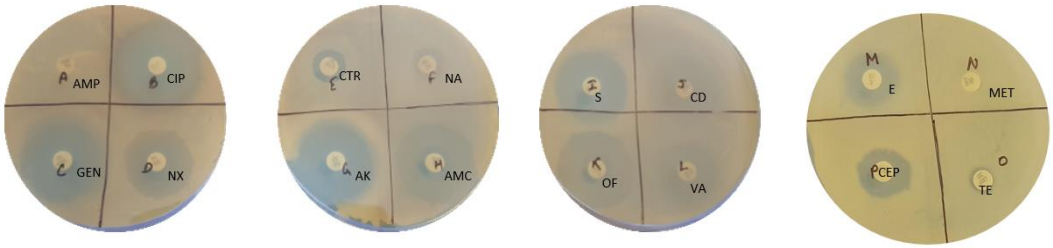
Isolate: LS 6



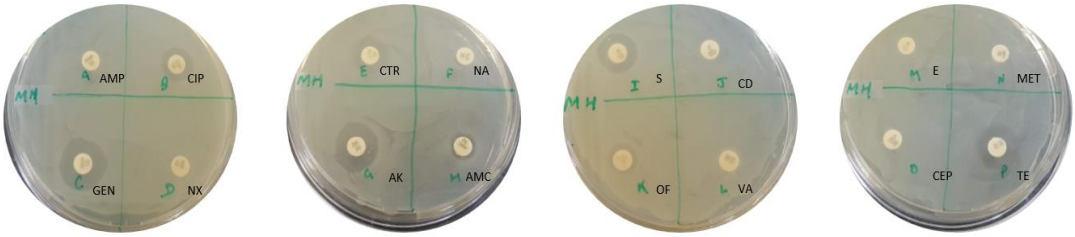
Isolate: LS 9



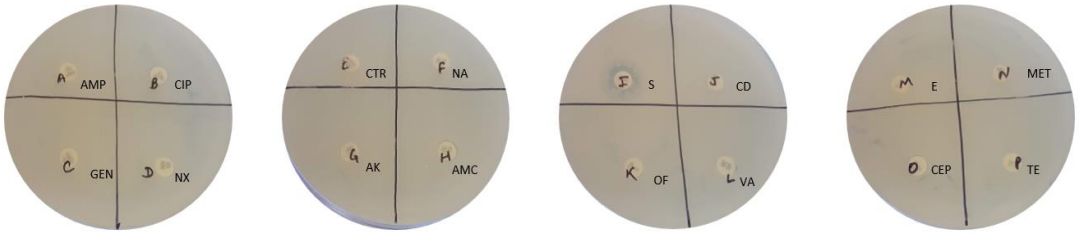
Isolate: LS 12



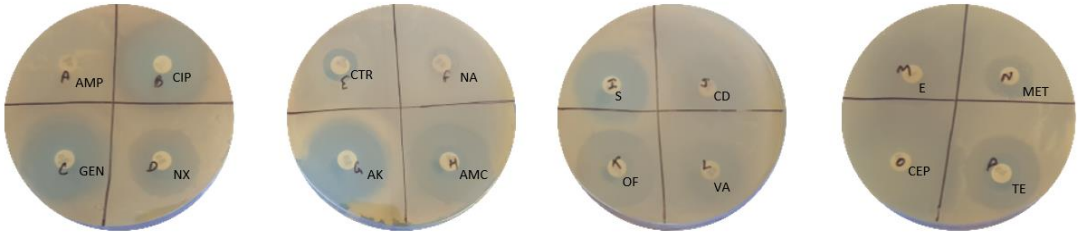
Isolate: LS 14



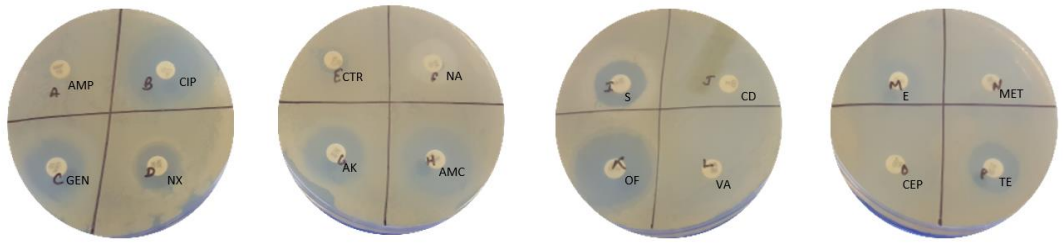
Isolate: LS 17



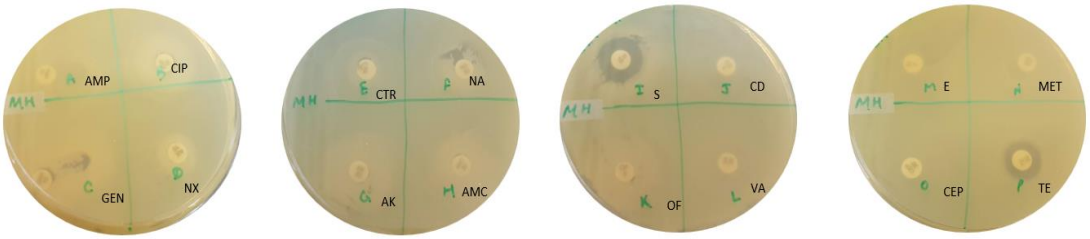
Isolate: LS 21



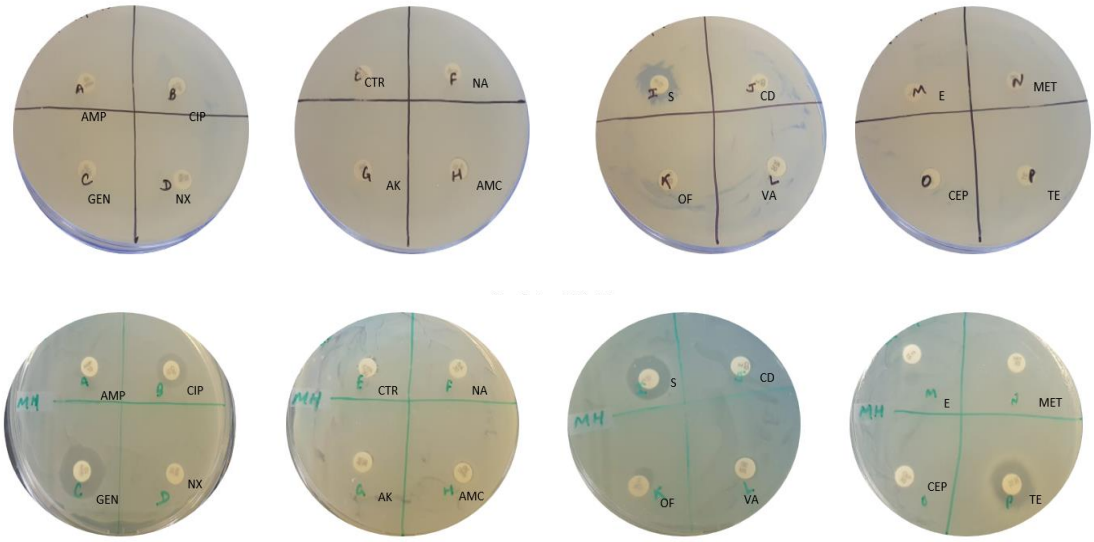
Isolate: SS 3



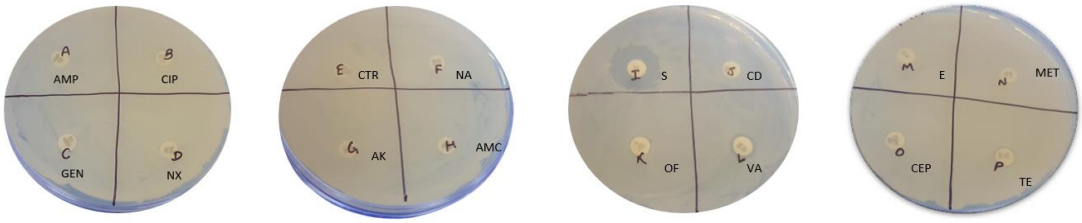
Isolate: SS 19



Isolate: LS 23



Isolate: SS 7



Isolate: SS 20

Fig. 5.15 Antibiotic sensitivity assay by disc diffusion of MDR isolates

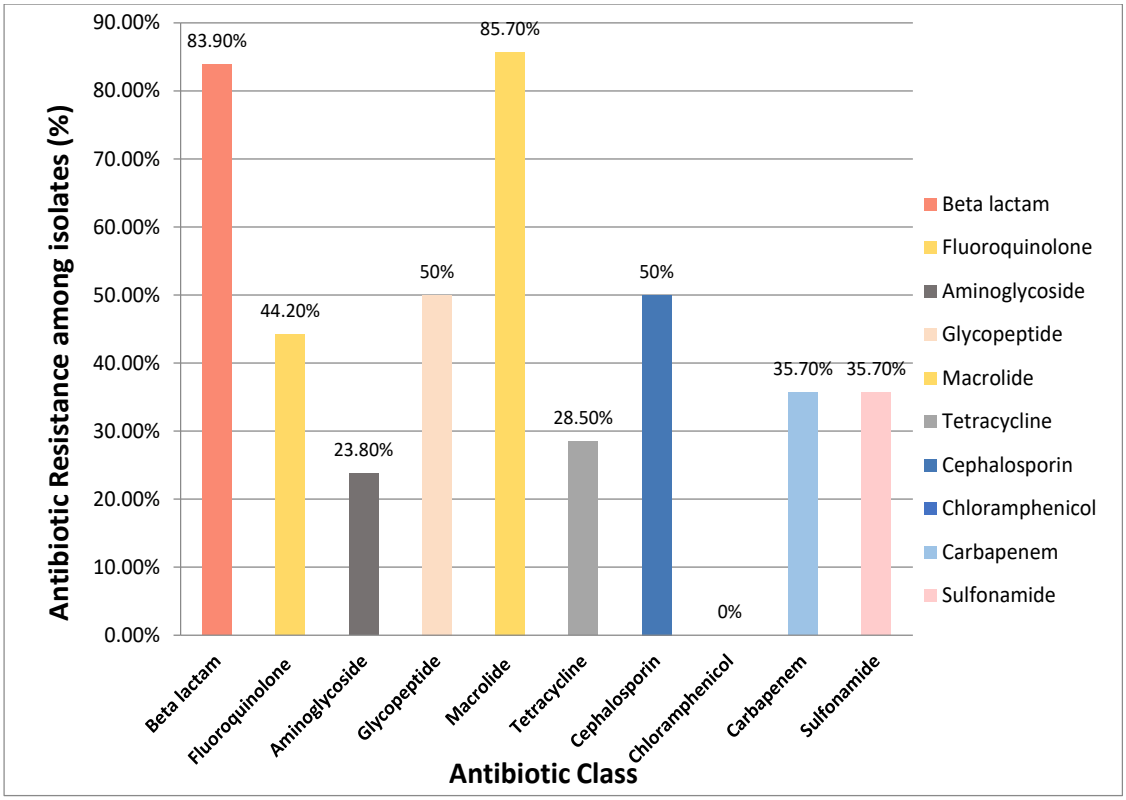


Fig. 5.16: "Antibiotic resistance patterns of bacteria isolated from wastewaters collected from rural and urban areas of West Bengal, India. (Graphical representation of % antibiotic resistance on y-axis plotted against various classes of antibiotics on x-axis

5.8 Genomic DNA isolation

The MDR and XDR isolates were subjected to genomic DNA isolation.

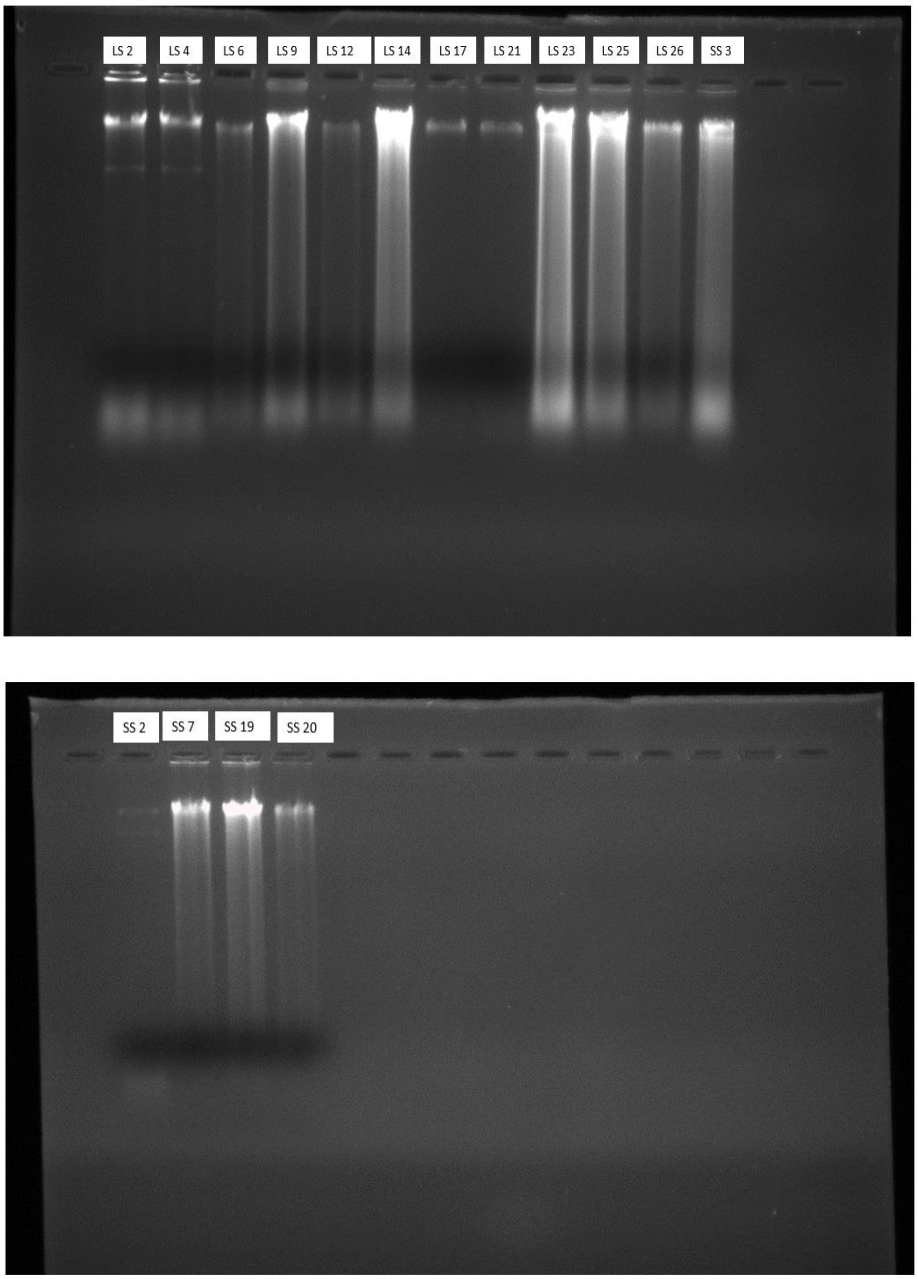


Fig. 5.17: Agarose gel electrophoresis for visualization of genomic DNA

5.9 PCR amplification of 16S rRNA gene

The genomic DNA of MDR and XDR isolates were subjected to PCR amplification.

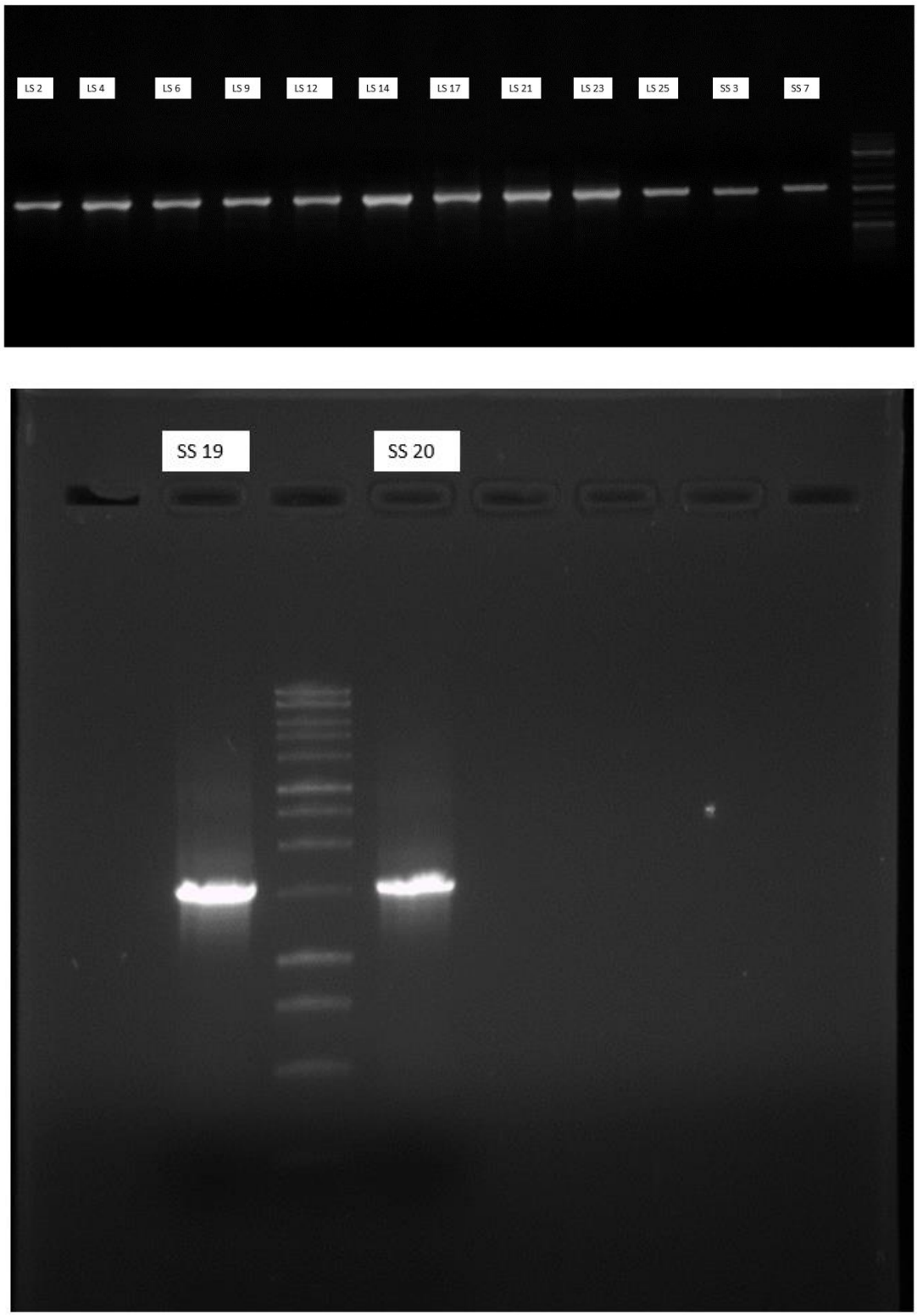


Fig. 5.18: PCR amplification of 16S rRNA gene

5.10 PCR product purification by agarose gel extraction

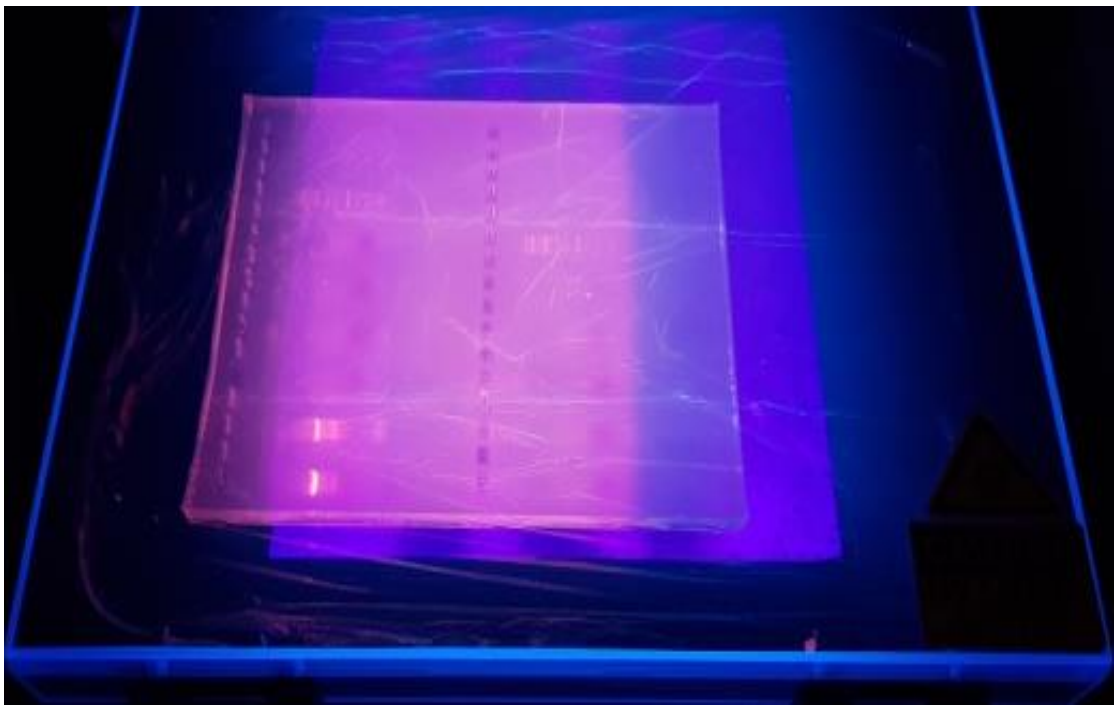


Fig.5.19: Purified PCR product as observed under UV light

5.11 Phylogenetic analyses

"Phylogenetic profiling revealed the presence of 4 distinct phylogenetic clusters:

- The largest cluster had 5 individual OTUs (LS 4, SS 7, LS 17, LS 9, SS 19). In this group, all the isolates showed resistance against AMP, NAL, Cd, VAN and MET antibiotics. It is interesting to note that this group contains isolates obtained from different geographical and sampling conditions which indicate the possibility of genetic exchange amongst allochthonous species (Lupo et al., 2012).
- The cluster containing isolates LS 6, LS 2, LS 21 show resistance against a wide range of antibiotics such as beta lactams, fluoroquinolones, aminoglycoside, macrolides, tetracycline, cephalosporins, carbapenems and sulphonamides, evolving probably through Darwinian forces (Gullberg et al., 2011).
- The sister group with OTUs obtained from different sampling conditions such as LS 14, SS 20, LS 12 show resistance against beta lactams and cephalosporins

both groups inhibiting bacterial cell wall synthesis.

- The cluster with OTUs LS 23, SS 3 and LS 25 contains halophilic organisms with resistance against some common antibiotics such as beta lactams and cephalosporins.
- LS 2 and LS 6 belong to the sister clades and exhibit similar antibiotic resistance patterns viz. resistance against beta lactams, fluroquinolones, aminoglycosides, macrolides, tetracycline, cephalosporins and sulfonamide. LS 25 and SS 3 show resistance against beta lactams and glycopeptides again with the common mode of action, i.e., inhibition of cell wall synthesis. They are closely related members with respect to similarities in 16S rRNA gene sequences which justifies the observations of Martinez (2009), that evolutionarily related bacteria have greater chance of being selected for in polluted environments either due to the presence of antibiotics or by the process of co selection of other pollutants."

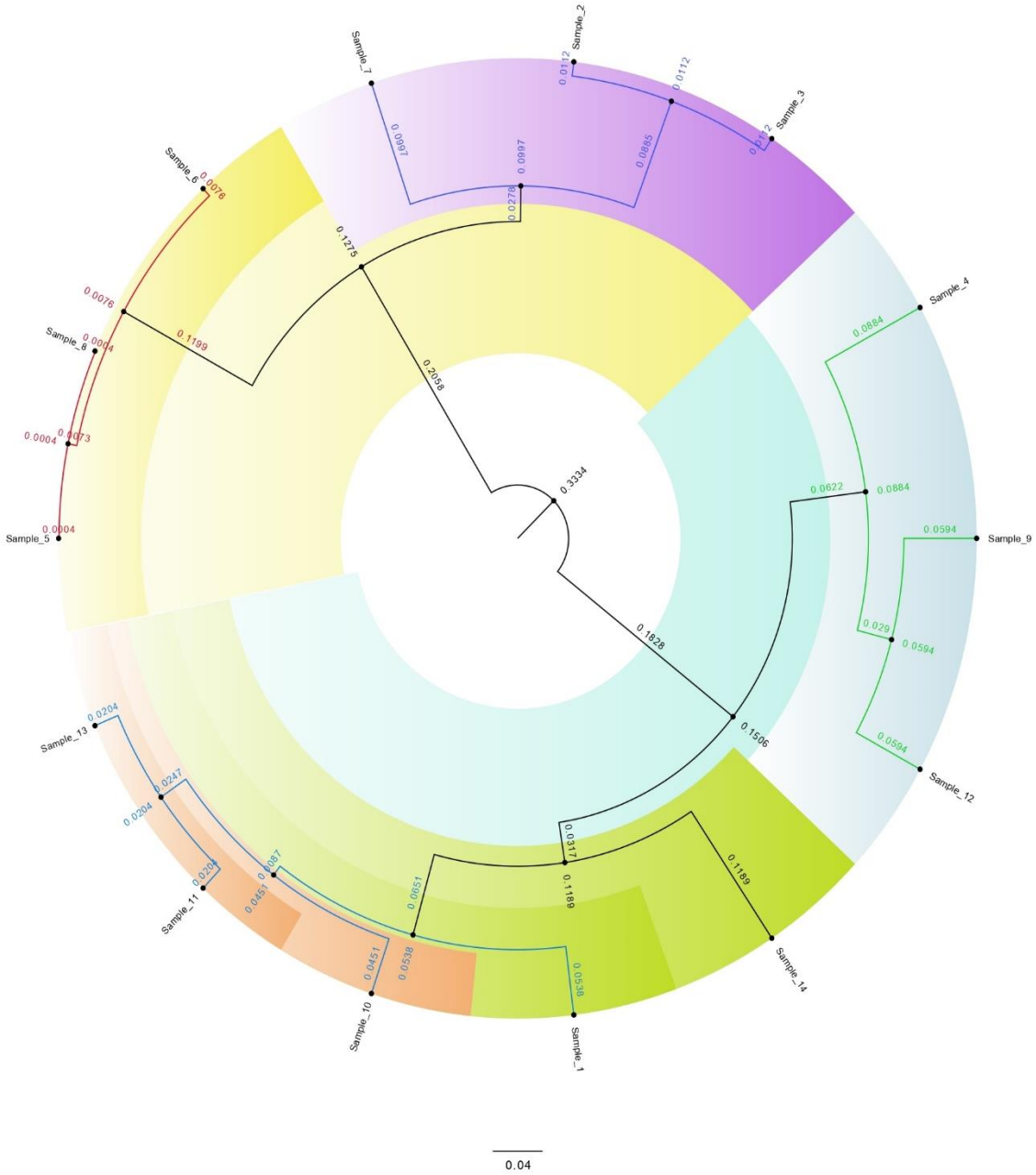


Fig. 5.20: "Phylogenetic analysis. Four distinct clades were identified in the phylogenetic tree (Clade 1 -LS 4, SS 7, LS 17, LS 9, SS 19; Clade 2- LS 6, LS 2, LS 21; Clade 3- LS 14, SS 20, LS 12; Clade 4- LS 23, SS 3

Sample ID	Isolate	Antibiotics resistant pattern
LS 2	*Escherichia coli*	AMP, CIP, GEN, NOR, CTR, NAL, AK, AMC, Cd, OFX, VAN, ERY, MET, Cf, TET, DO, CTX, IPM, CXM, LE, COT, CX
LS 4	*Shigella flexneri*	AMP, CIP, NAL, AMC, Cd, E, MET, Cf, CXM
LS 6	*Klebsiella pneumoniae*	AMP, CIP, GEN, CTR, AK, AMC, OFX, NAL, AMC, Cd, E, MET,Cf, TET, DO, CTX, IPM, CXM, COT
LS 9	*Escherichia coli*	AMP, NOR, NAL, Cd, MET, Cf
LS 12	*Bacillus safensis*	AMP, Cd, ERY, MET, Cf, CXM
LS 14	*Bacillus australimaris*	AMP, NAL, AMC, Cd, VAN, ERY, MET, Cf, CX
LS 17	*Escherichia coli*	AMP, NAL, Cd, OFX, VAN, ERY, MET
LS 21	*Comamonas aquatica*	AMP, GEN, NOR, CTR, NAL, AK, AMC, Cd, OFX, ERY, MET, Cf, TET, DO, CTX, IPM, CXM
LS 23	*Lysinibacillus fusiformis*	AMP, CIP, GEN, NOR, CTR, NAL, AK, AMC, Cd, OFX, ERY, MET, Cf, TET, CTX, IPM, CXM, COT
LS 25	*Oceanobacillus caeni*	AMP, GEN, NOR, CTR, NAL, AK, AMC, Cd, OFX, VAN, ERY, MET, Cf, CTX, IPM, CXM, COT
SS 3	*Terribacillus halophilus*	AMP, VAN, ERY, Cf
SS 7	*Citrobacter freundii*	AMP, CIP, CTR, NAL, Cd, OFX, ERY, MET, Cf, TET
SS 19	*Comamonas aquatica*	AMP, NAL, Cd, VAN, MET
SS 20	*Bacillus pumilus*	AMP, CIP, CTR, NAL, AMC, Cd, MET, Cf, CXM

Table 5.10: Antibiotic resistance patterns of various isolates

5.12 Whole Genome analyses

Whole genome analyses of the XDR *E. coli* isolate revealed the abundance of ARF profiles in the genome. ARGs present are involved in various mechanisms of antibiotic resistance such as efflux pumps, protein modification, enzyme inactivation etc.

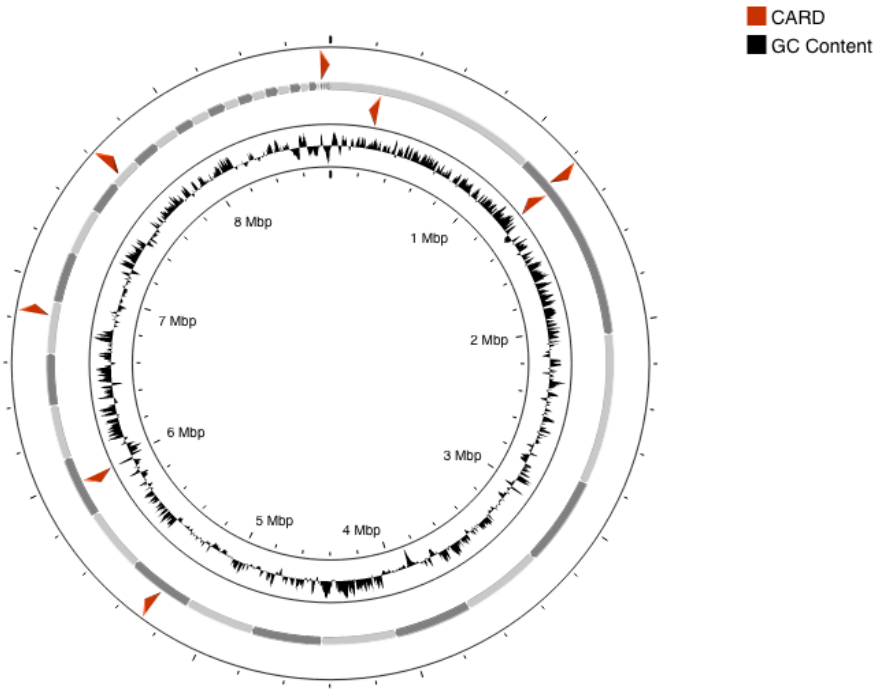


Fig. 5.21 ARG Profiles in the genome

AMR Mechanism	Genes
Antibiotic activation enzyme	KatG
Antibiotic inactivation enzyme	APH(3')-II/APH(3')-XV, L1 family, LCR/NPS family, OXA-48 family
Antibiotic target in susceptible species	Alr, Ddl, dxr, EF-G, EF-Tu, folA, Dfr, folP, gyrA, gyrB, inhA, fabI, Iso-tRNA, kasA, MurA, rho, rpoB, rpoC, S10p, S12p
Antibiotic target protection protein	QnrB family
Antibiotic target replacement protein	fabV
Efflux pump conferring antibiotic resistance	EmrAB-OMF, EmrAB-TolC, MacA, MacB, MdtABC-OMF, MdtABC-TolC, MexEF-OprN, MexEF-OprN system, MexJK-OprM/OpmH, MexVW-OprM, TolC/OpmH
Gene conferring resistance via absence	gidB
Protein altering cell wall charge conferring antibiotic resistance	GdpD, PgsA
Protein modulating permeability to antibiotic	OccD4/OpdT, OccD6/OprQ, OccK5/OpdH, OprB family, OprD family, OprF
Regulator modulating expression of antibiotic resistance genes	OxyR

Table 5.11: ARG conferring to various mechanisms of antibiotic resistance

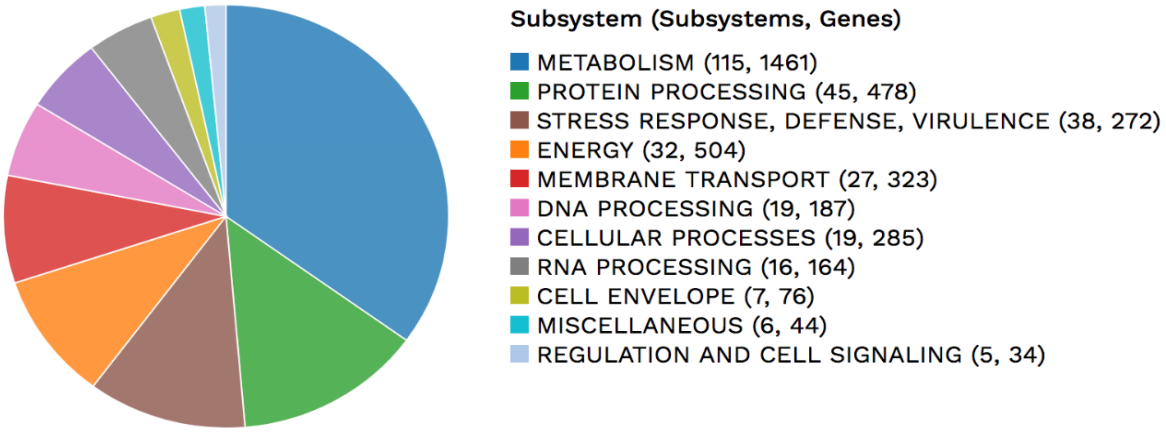


Fig. 5.22: Genes and modules

5.13 Virtual screening

Results from the screening of the compounds for their drug likeliness and bioavailability revealed that most of the compounds analyzed were suitable as potential leads since they did not violate the Lipinski's Rule of five (Lipinski et. al, 2001) by more than one parameter. However, Hexacosyl hexadecanoate was found to have a higher molecular weight than the acceptable limit and would need suitable chemical modifications if selected. The compounds were then docked with the selected targets and their atomic contact energies were noted. For 5TKW the best combinations of energy and area of interaction was found to be with Beta sitosterol; for 2XCQ it was Kaempferol and for 4E81; Hexacosyl hexdecanoate proved to be the best interacting molecule.

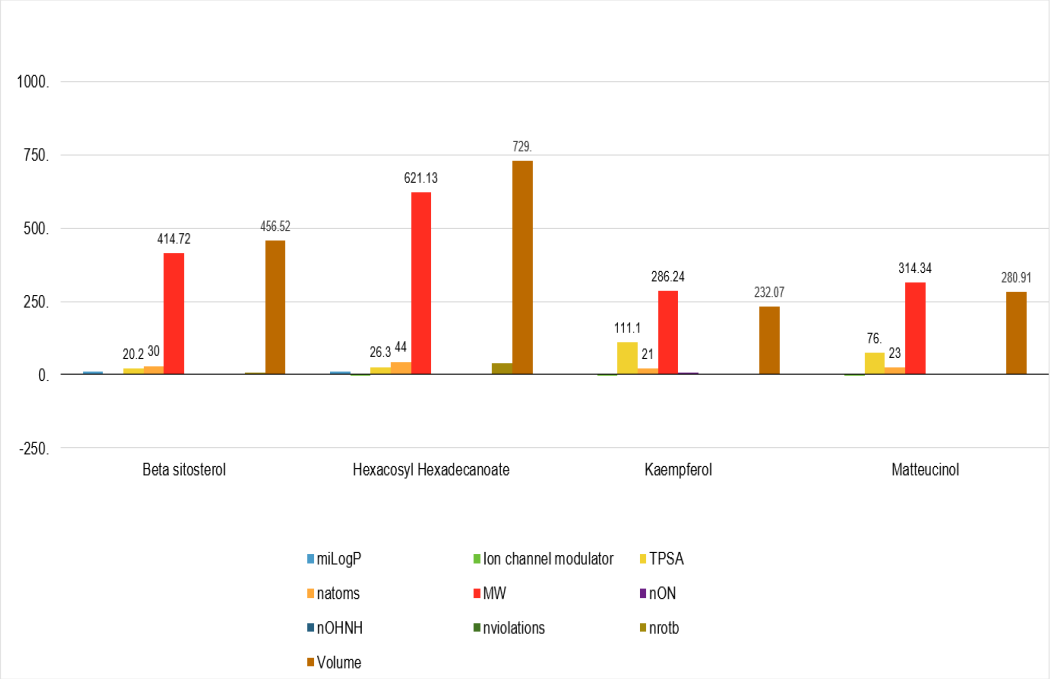


Fig. 5.23: Molinspiration data

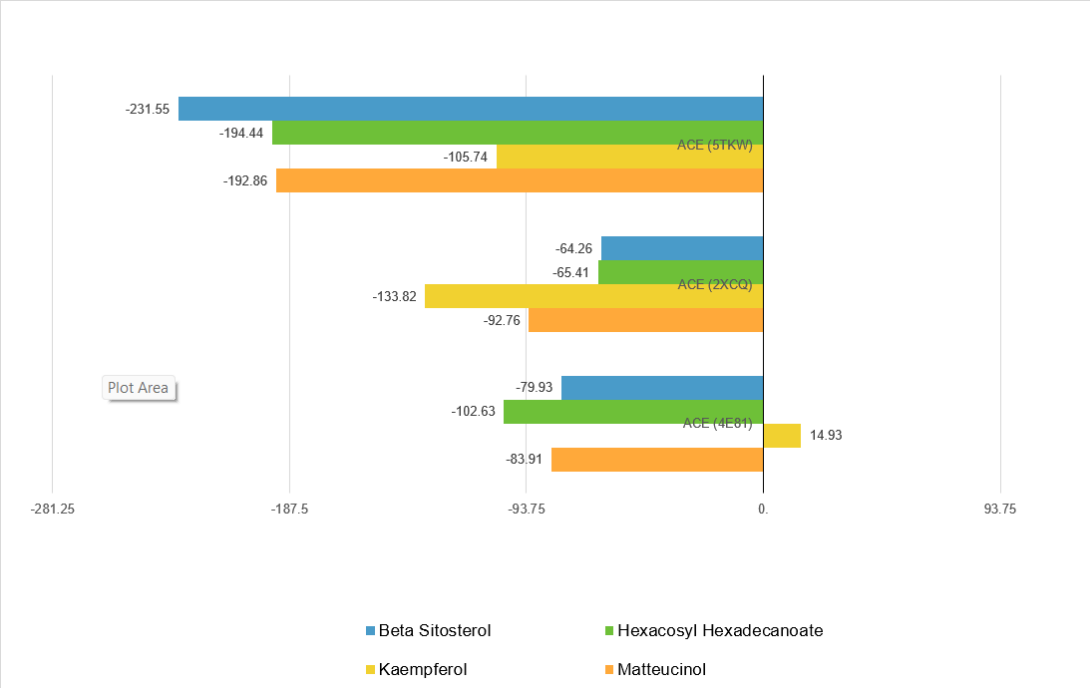


Fig. 5.24: Atomic Contact Energy (ACE) values

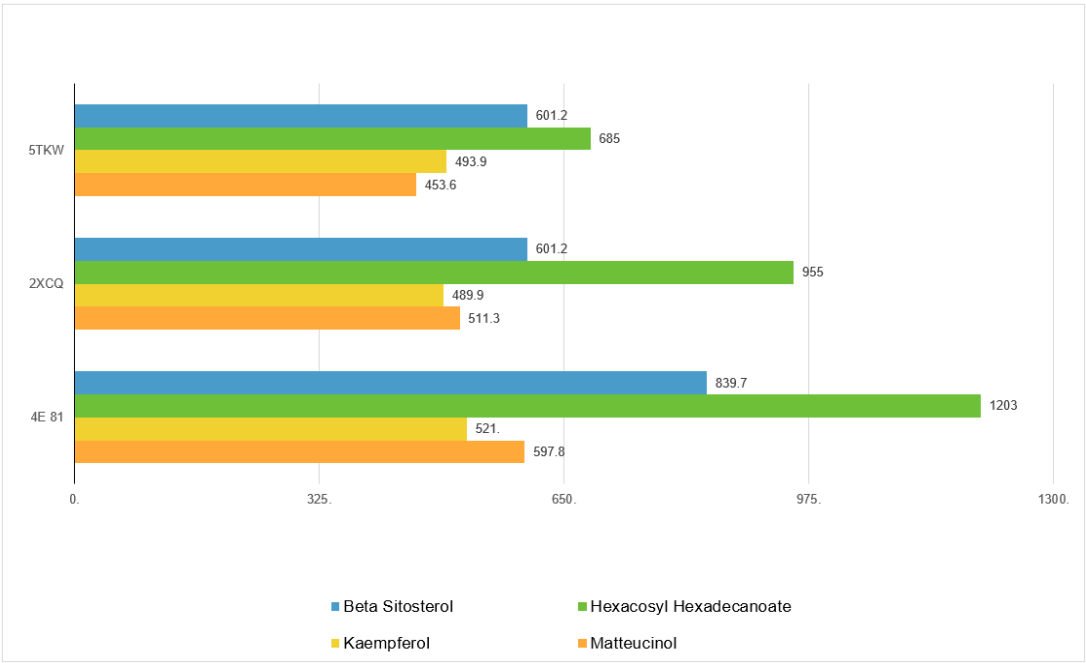


Fig. 5.25: Area of docking

Molecular docking results:

The analyses of the interaction complexes of the best results revealed that hydrogen bonded and non-bonded interactions were together regulating the complex formation. In case of 5TKW- Beta sitosterol interaction 2 hydrogen bond(s) having distance of 2.77 angstrom and 3.02 angstrom along with 49 Van der Waals interactions were identified whereas 2XCQ-Kaempferol interactions revealed 2 hydrogen bonds with a distance of 2.82 angstrom and 2.91 angstrom and 12 Van der Waals contacts.

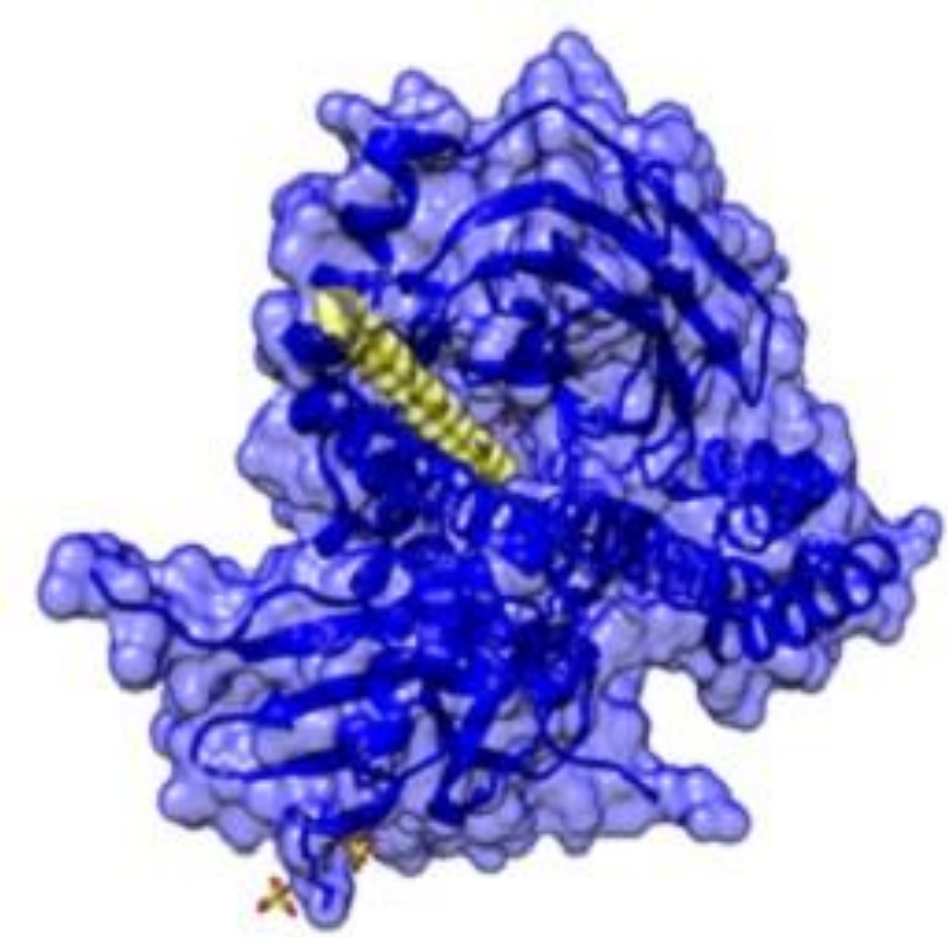


Fig. 5.26 (a): 5TKW- Beta sitosterol: Docked compound

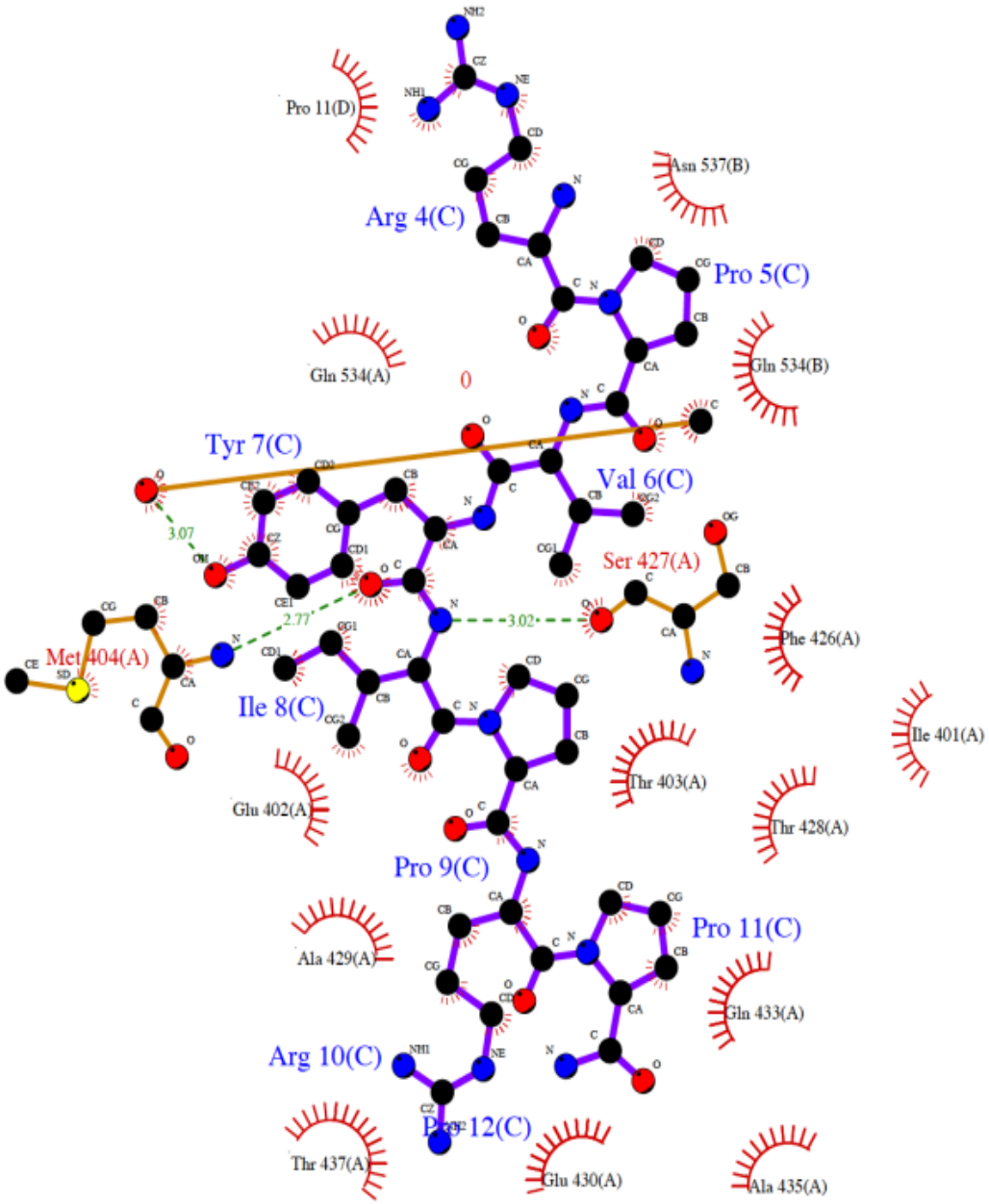


Fig. 5.26 (b): 5TKW- Beta sitosterol: Hydrogen bond and Van der Waals interactions

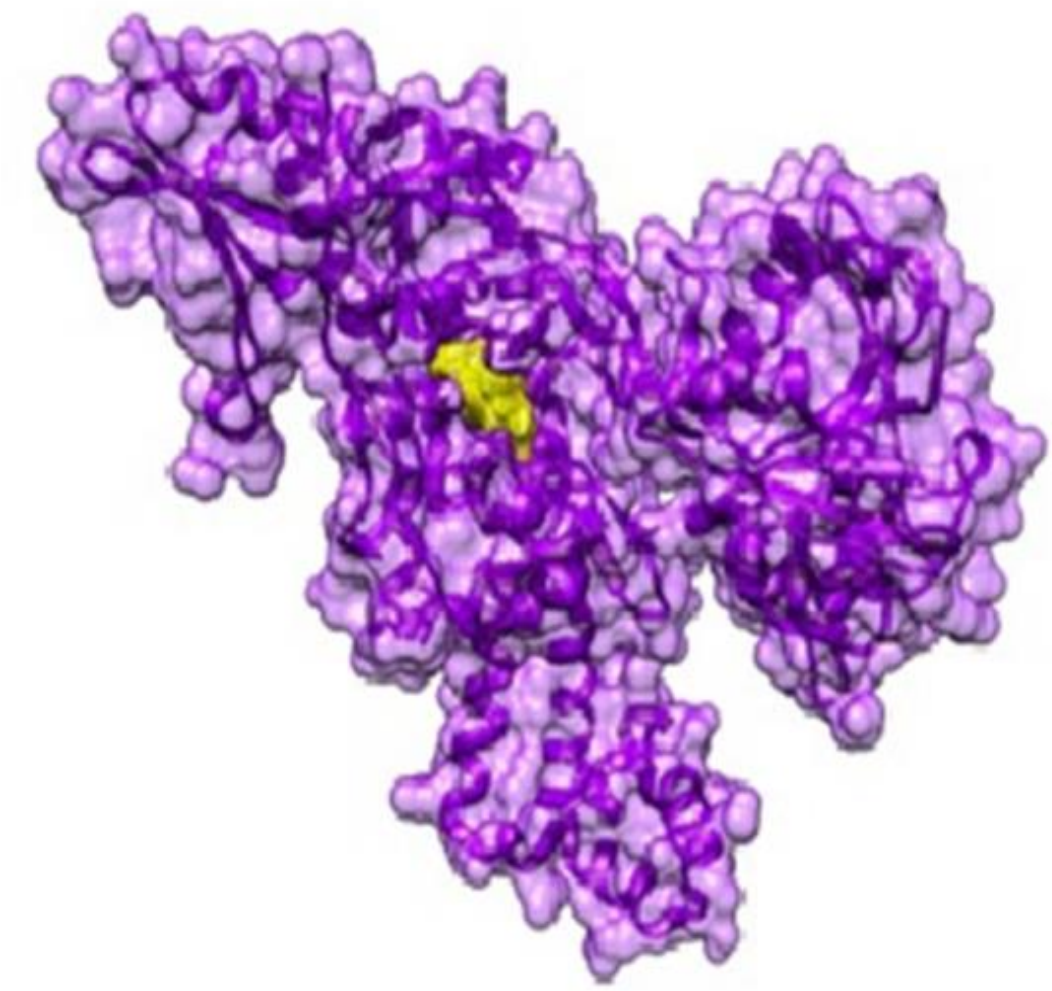


Fig. 5.27 (a): 2XCQ- Kaempferol: Docked compound

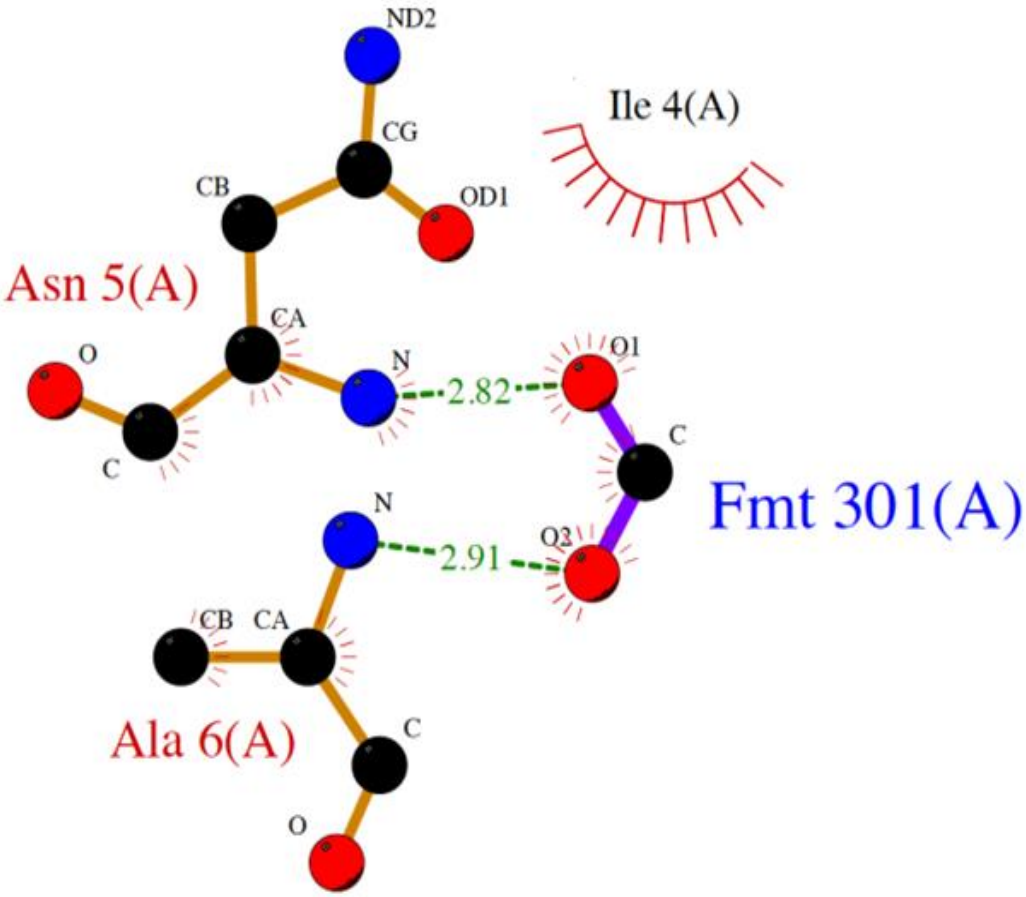


Fig. 5.27 (b): 2XCQ- Kaempferol: Hydrogen bond and Van der Waals interactions

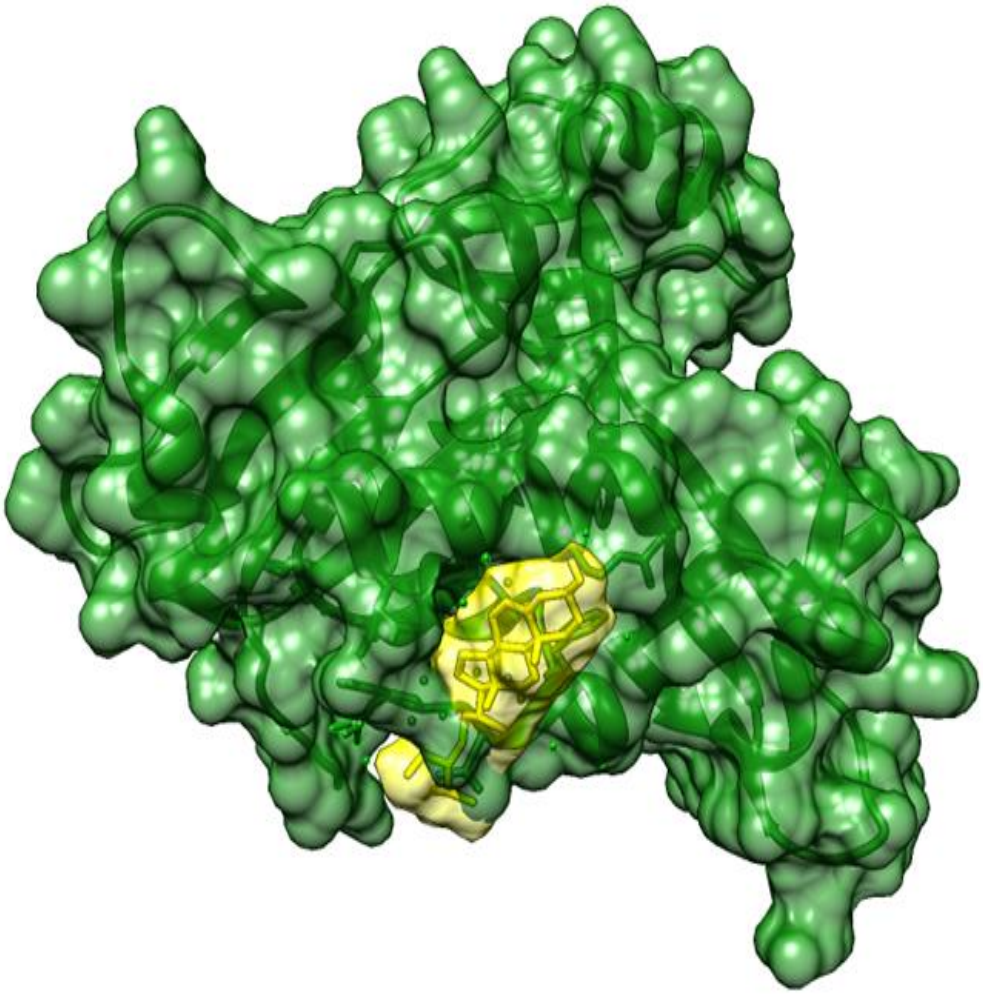


Fig. 5.28 (a): 4E81- Hexacosyl hexadecanoate: Docked compound

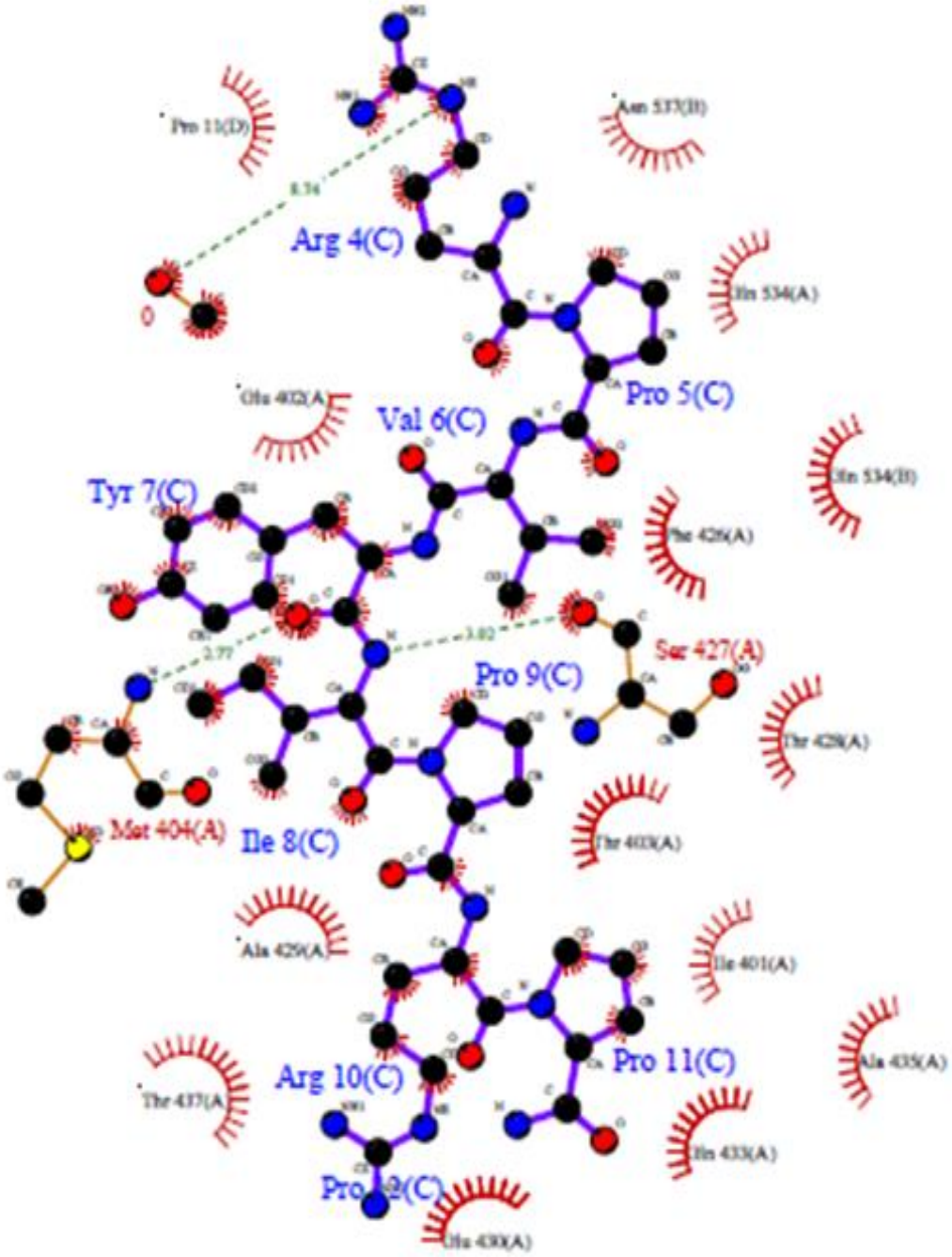


Fig. 5.28 (b) 4E81- Hexacosyl hexadecanoate:Hydrogen bond and Van der Waals interaction

6. DISCUSSION

6. Discussion

The environment has a role in both evolution and transfer of resistance. However, the limited understanding of the exact whereabouts and critical conditions which lead to the occurrence of new MDR and XDR bacteria in the environment is a major cause of concern. It is very crucial to understand the importance of site selection in the isolation and identification of resistant bacteria. The surveillance strategies whether isolate based or gene based should focus primarily on the site of sample selection and necessary steps should be taken to ensure sample collected should either represent the average resistome or average abundance of selected genes across the microbiota, respectively. Further genome sequencing of resistant strains can help us to visualize the bigger picture of antibiotic resistance in the environment. The control measures and the mitigation strategies can be efficiently employed only if the extent of spread of antibiotic resistance is clear.

It is evident through this thesis that wastewater-based surveillance can act as markers for routine studies after a particular interval whether monthly or annually. The samples which are collected as close to the emission source as possible are of vital importance because they represent the larger populations. The monitoring of raw wastewater is very promising because it contains pooled faecal bacteria. The broad coverage provided by wastewater-based surveillance is also advantageous because it demands low resources and can also give us an early warning for the early spread of resistance from a previously unknown location. The sequencing of certain MDR and XDR bacteria in this study provides the species-specific phenotypic data with certainty which can help us to list priority pathogenic and non-pathogenic resistant bacteria empirically for future study.

Metagenomics based approaches are critical in monitoring of regional, temporal, and spatial trends in antibiotic resistance. The ease of standardization and no association with ethical issues which may occur in case of clinical surveillance make it a vital tool in resistance analyses. This study is based on culture dependent and metagenomics to understand the environmental resistome. The resistance genes (antibiotic and metal) have also been determined in bulk environmental samples.

To gain a comprehensive understanding of resistomes, both molecular and cultivation methods are required. Our findings revealed discrepancies between the results obtained

from culture-based methods and metagenomics, indicating variations at both the species and genus levels.

This thesis focuses on resistant bacteria present in various environmental matrices. The previous studies have majorly relied on the identification ARGs because of it is difficult to culture environmental bacteria with standard methods and moreover identify trends of resistance. The isolate-based study provides insights into both phenotype and context since it has also been combined with whole-genome sequencing of XDR and MDR bacteria.

XDR *Escherichia coli*

"The Antimicrobial Resistance Collaboration recently provided the most comprehensive global estimate of the burden of antimicrobial resistance (AMR) across 23 pathogens, 88 pathogen-drug combinations and 204 countries. It was estimated that there were 4.95 million deaths in 2019 associated with bacterial AMR, including 1.27 million deaths attributable to bacterial AMR. *Escherichia coli* (*E. coli*) was the number one culprit pathogen, with 829,000 AMR associated deaths and 219,000 AMR attributable deaths. The ability of *E. coli* to colonize different environments, including the gut of humans and animals, has provided this organism with the evolutionary advantage to acquire antibiotic resistance traits from other bacteria within its environment, as well as to be easily transmitted via the faecal-oral route."

Our findings: "It was found that *E. coli* isolate (LS 2) was resistant against 22 antibiotics out of 24 antibiotics tested which is in conformation with the public data released by ICMR. The percentage of resistance was higher in *E. coli* isolates compared to other isolates. Some *E. coli* isolates (LS 2, LS 9, LS 17) showed variable pattern of susceptibility which may be attributed to its higher relative abundance in fecal contaminated environments or increased competence towards receiving resistant gene containing foreign plasmids (Santos-Lopez et al., 2019)."

Previous reports: "In 2020, a WHO report showed that the rate of ciprofloxacin-resistant *E. coli* ranges in over 30 countries and regions is about 8.4%–92.9%.

Prevalence of multidrug-resistant and extended-spectrum beta-lactamase-producing Escherichia coli in urban community wastewater was also reported in Germany. (Schemiege et al., 2021).

A nationwide study of more than 11,000 *E. coli* bloodstream infections in Israel, detected a higher case fatality rate with multi-drug resistant (MDR) versus non-MDR

E. coli (47% versus 28%) (Feldman et al., 2022). Recent studies have shown that *E. coli* resistance rates vary among various regions.

An analysis of municipal and hospital wastewater in Japan found a high number of multidrug-resistant *Escherichia coli* strains wherein the isolates from both the hospital and municipal wastewater showed a high resistance rate to AMP, CXM, CTX, CIP, and cefepime. (Shibuki et al., 2023)."

XDR *Klebsiella pneumoniae*

"K. pneumoniae is one of the commonest isolates in both hospital and community acquired infections (Caneiras *et al.*, 2019). MDR and carbapenem resistant *K. pneumoniae* has become a major therapeutic challenging scenario in several countries due to the lack of alternative existing antibiotics." *E. coli* and *K. pneumoniae* have been declared as indicator bacteria in AMR surveillance in the environment (Anjum et al. 2021).

Our findings: "In our study, *K. pneumonia* isolate (LS 6) exhibits resistance against beta lactams, fluoroquinolones, aminoglycoside, macrolides, tetracycline, cephalosporins, carbapenems and sulphonamide."

Previous reports: "*K. pneumoniae* represent the commonest multidrug-resistant (MDR) pathogens that exhibit ESBL, carbapenems and quinolone resistance (Castanheira et al. 2021; Cheng et al. 2018; Harmon et al. 2019; Klein et al. 2018)."

"In Spain, third generation cephalosporin-resistant *Klebsiella pneumoniae* were identified in clinical and wastewater samples (Rocha et al., 2022)."

"Sewage-based surveillance showed the presence of *Klebsiella pneumoniae* resistant against last resort antibiotics such as colistin, cefotaxime and tigecycline in the population in Bergen, Norway (Radisic et al., 2023)."

MDR *Shigella flexneri*

"Shigella spp. are an important group of waterborne pathogens worldwide. They are the most common cause of diarrheal disease and have remained a major pathogen responsible for increased rates of morbidity and mortality caused by dysentery each year around the globe, particularly affecting children aged <5 years in developing countries. (Puzari et al., 2018). *Shigella* spp. are resistant to most antibiotics, and drug treatment related to these bacteria is costly, time-consuming, and sometimes problematic, particularly in areas with limited medical care. About half the strains of *Shigella* in many parts of the world are now resistant to multiple drugs.

Our findings: In our study we found that *Shigella flexneri* isolate (LS 4) showed variable resistance towards beta lactams, fluoroquinolones, macrolides and cephalosporins.

Previous reports: The increase of multidrug resistance (MDR) in *Shigella* spp. against third-generation cephalosporins (TGC), azithromycin and fluoroquinolones, is a major issue in developing countries which makes the treatment of shigellosis more difficult (Ma et al., 2018).

Several reports from around the world specially, India, Canada, Israel, Argentina, Turkey, Lebanon, Iran, China, Japan, and South Korea, have reported *Shigella spp.* harbouring different types of ESBL genes (Ranjbar *et al.*, 2019).

There has been a rampant increase in XDR Shigellosis in the United States. In 2022, about 5% of *Shigella* infections reported to CDC were caused by XDR strains, compared with 0% in 2015. CDC defines XDR *Shigella* bacteria as strains that are resistant to all commonly recommended empiric and alternative antibiotics — azithromycin, ciprofloxacin, ceftriaxone, trimethoprim-sulfamethoxazole (TMP-SMX), and ampicillin."

MDR *Lysinibacillus fusiformis*

"Several strains of the commonly occurring *Lysinibacillus fusiformis* have been isolated from multiple environments including agriculture soil and factory wastewater (Ahmed et al., 2007). It is recognized as a ubiquitous environmental bacterium and has been isolated from wastewater, plants, and soil. (Wang et al., 2010).

Our findings: In our study, *L. fusiformis* (LS 23) was found to be resistant against beta lactams, fluoroquinolones, macrolides, tetracycline, cephalosporins and sulphonamide. This is the first report of multidrug resistance in *L. fusiformis.*

Previous reports: *L. fusiformis* has been reported to be sensitive to tetracycline previously (Priest et al., 1988). A new *Lysinibacillus fusiformis* strain was isolated from from Metlaoui Phosphate Laundries Wastewater and was found to be resistant against penicillin. (Taeib et al., 2021).

MDR *Bacillus pumilus*

Bacteria of the *Bacillus pumilus* clade are ubiquitous in a wide variety of terrestrial and marine environments, ranging from stratospheric air to deep-sea sediments and from soil to living beings (Shivaji *et al.*, 2006; Liu *et al.*, 2013; Branquinho et al., 2015).

Our findings: The following species belonging to *B. pumilus* clade: *B. pumilus* (SS 20), *B. safensis* (LS 12) and *B. australimaris* (LS 14) were identified in this study. The

antibiotic resistance patterns of the sister clade isolates are quite similar in nature with resistance shown against beta lactams, fluoroquinolones, macrolide and cephalosporins. This presents an interesting insight into the evolutionary link of antibiotic resistance genes across various species.

Previous reports: There are no previous reports on drug resistance of bacteria belonging to the *Bacillus pumilus* clade.

MDR *Comamonas* spp.

Comamonas species are occasional human pathogens found in contaminated environments (Farooq *et al*., 2017). They have been isolated from a broad variety of environments, including water, aircraft water, soil, plants, and animals (Zhong et al., 2015; Handschuh et al., 2017; Xiong et al., 2011; Andrade et al., 1997; Pavone et al., 2021). Even though *Comamonas* spp. are thought of as being of low virulence, they have caused harmful health conditions in many healthy individuals and even death in patients with underlying conditions. Antimicrobial treatment of infections associated with these species, in general, is not very difficult; however, it can become an issue in the future because some strains are already resistant to different classes of antibiotics. Therefore, these pathogens should be considered of such importance that they should be included in the hospital screening programs.

Our findings: We have identified two *C. aquatica*, one each from both the samples (LS 21 and SS 19), which show variable resistance against beta lactams, fluoroquinolones, and glycopeptide class of antibiotics.

Previous reports: In a study by Hem et al., 2022, 32 *Comamonas. denitrificans* and 5 *C. testosteroni* from wastewater, 1 *C. denitrificans* from a wetland, and 1 *C. aquatica* from a lake with public access were sequenced. All were found to be resistant to carbapenem antibiotics.

MDR *Oceanobacillus caeni*

Oceanobacillus caeni was first isolated in South Korea as a component of activated sludge in a Bacillus-dominated wastewater treatment plant (Nam *et al.*, 2008).

Our findings: In our study the isolate (LS 25) was obtained from rural wastewater and it showed variable resistance against beta lactams, fluoroquinolones, macrolides, cephalosporins and sulphonamide.

Previous reports: The pathology and antibiotic resistance patterns of this species have not been conclusively studied before.

MDR *Citrobacter freundii*

Citrobacter freundii is a frequent cause of nosocomial infections and a known cause of diarrheal infections, has increasingly become multidrug resistant (Liu *et al.*, 2018). MDR strains of *C. freundii* are more commonly acquired among high-risk patients such as neonates, immunocompromised and elderly people

Our findings: *C. freundii* isolate (SS7) was identified and showed resistance against beta lactams, fluoroquinolones, aminoglycoside, macrolides, cephalosporins and tetracycline.

Previous reports: A MDR *Citrobacter freundii* strain was isolated from a wastewater treatment plant in China. It was found that a total of 13 antibiotic-resistance genes (ARGs) that confer resistance to eight different antibiotic groups were encoded by this strain (Jiang et al., 2019).

MDR *Terribacillus halophilus*

Terribacillus halophilus was originally isolated from field soil in Japan (An *et al*., 2007).

Our findings: The isolate (SS 3) showed variable antibiotic resistance patterns against beta lactams, glycopeptides, macrolides, and cephalosporin.

Previous reports: This is the first report of occurrence of antibiotic resistance in this species."

OVERALL SUMMARY

Overall Summary

- Cd, Pb, Hg were found to be higher than the permissible limits in both effluents. (As per EPA Rules, 1996).
- COD was found to be higher than the permissible limits.
- *Prevotella*, *Bacillus*, *Clostridium* are the dominant common genera in both datasets as per metagenomics-based analysis.
- Comprehensive Metagenomic Profiling was performed and data submitted to SRA(NCBI) with accession numbers: SAMN11571463 and SAMN11571474.
- Species richness was higher in urban dataset (SS) than that of rural dataset (LS).
- High macrolide resistance (85 %) and beta lactam resistance (83.9 %) was observed with all the isolates being resistant to Ampicillin.
- 14 MDR isolates were identified and deposited in GENBANK

SUB8757948 ls-04 MW380613
SUB8757948 ls-06 MW380614
SUB8757948 ls-02 MW380615
SUB8757948 ls-23 MW380616
SUB8757948 ls-14 MW380617
SUB8757948 ss-20 MW380618
SUB8757948 ls-21 MW380619
SUB8757948 ls-12 MW380620
SUB8757948 ss-03 MW380621
SUB8757948 ss-07 MW380622
SUB8757948 ls-17 MW380623
SUB8757948 ls-25 MW380624
SUB8757948 ls-09 MW380625
SUB8757948 ss-19 MW380626

- The microorganisms present in rural sample (LS) have higher number of multidrug resistance genes, multiple antibiotic resistance and stress resistance proteins than that in urban sample (SS).
- The new knowledge that has been generated using the analysis carried out has enabled us to identify some very important MDR strains that are prevalent in the sampling sites as well as are part of the urban and rural resistant reservoir of the state. This data can be taken up by the Health Department to monitor the prevalence of these organisms on a timescale basis over the year.
- Added to this, we have been able to devise a Pathogenic Load prediction pipeline using taxon set enrichment analysis which will surely benefit us in predicting disease prevalence using taxon set identification as a low cost and low time scale alternative to the entire process.
- The novel natural ligands identified to be potential leads against the key disease modifier proteins can serve as very important landmarks for future drug discovery programmes which will benefit the common man in tiding over the menace of antibiotic resistance.

CONCLUSION

Conclusion

It is important to compare environmental resistance data with reliable clinical data as a benchmark. This comparison enables towards the accuracy and quality of the resistance information and helps to establish significant associations between environmental and clinical resistance profiles. Based on current evidence, the environment plays a critical role in transfer of antibiotic resistance between pathogenic and non-pathogenic bacteria. It also acts a reservoir for intermediate habitat of resistant bacteria.

The new reports mentioned in this thesis above show occurrence of multi-drug resistance in various bacteria which is very alarming. These bacteria possess the ability to transfer the resistant genes to other bacteria thriving in the same environmental matrix. Therefore, it is very important to determine these environmental hotspots so that proper control measures can be taken. The key knowledge gaps need to be filled by building on existing systems and identification tools based on both culture-based and metagenomics-based approaches to determine the impact of wastewater discharge on environment and the habitants of the environment. These measures are cost-effective and the ease with which data can be compared can ensure the precision. In turn, this would enable improved surveillance of antibiotic resistance and the implementation of preventive measures for the future.

Docking of phytocompounds against proteins of multi-drug resistant bacteria can aid in the discovery of potential drug candidates. By identifying phytocompounds that can bind to and inhibit the activity of specific proteins involved in drug resistance, researchers can develop new strategies to combat antibiotic resistance and develop effective treatments against multi-drug resistant bacteria.

REFERENCES

7. References

1. Shlaes, D. M., & Bradford, P. A. (2018). Antibiotics-From There to Where?: How the antibiotic miracle is threatened by resistance and a broken market and what we can do about it. Pathogens and Immunity, 3(1), 19-43. doi: 10.20411/pai.v3i1.231.
2. Fleming, A. (1945). Penicillin. Nobel Lecture, December 11, 1945. Retrieved from http://www.nobelprize.org/nobel_prizes/medicine/laureates/1945/fleming-lecture.pdf
3. Antimicrobial Resistance Collaborators. (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. The Lancet, 399(10325), 629-655. doi: 10.1016/S0140-6736(21)02724-0
4. de Kraker, M. E., Stewardson, A. J., & Harbarth, S. (2016). Will 10 Million People Die a Year due to Antimicrobial Resistance by 2050? PLoS Medicine, 13(11), e1002184. doi: 10.1371/journal.pmed.1002184
5. Kawamura, K., Nagano, N., Suzuki, M., Wachino, J. I., Kimura, K., & Arakawa, Y. (2017). ESBL-producing Escherichia coli and Its Rapid Rise among Healthy People. Food Safety (Tokyo), 5(4), 122-150. doi: 10.14252/foodsafetyfscj.2017011
6. Manesh, A., Varghese, G. M., CENDRIC Investigators and Collaborators. (2021). Rising antimicrobial resistance: an evolving epidemic in a pandemic. The Lancet Microbe, 2(9), e419-e420. doi: 10.1016/S2666-5247(21)00173-7
7. Taneja, N., & Sharma, M. (2019). Antimicrobial resistance in the environment: The Indian scenario. Indian Journal of Medical Research, 149(2), 119-128. doi: 10.4103/ijmr.IJMR_331_18
8. Peterson, E., & Kaur, P. (2018). Antibiotic Resistance Mechanisms in Bacteria: Relationships Between Resistance Determinants of Antibiotic Producers, Environmental Bacteria, and Clinical Pathogens. Frontiers in Microbiology, 9, 2928. doi: 10.3389/fmicb.2018.02928
9. van den Honert, M., Sannettha, P.A.G., and Louwrens H.C. (2020). A Preliminary Study: Antibiotic Resistance Patterns of *Escherichia coli* and *Enterococcus* Species from Wildlife Species Subjected to

Supplementary Feeding on Various South African Farms" *Animals* 10, no. 3: 396. https://doi.org/10.3390/ani10030396

10. Vasilachi, I. C., Asiminicesei, D. M., Fertu, D. I., & Gavrilescu, M. (2021). Occurrence and Fate of Emerging Pollutants in Water Environment and Options for Their Removal. Water, 13(2), 181. doi: 10.3390/w13020181
11. Larsson,D.G.J., Flach, C.F. (2022). Antibiotic resistance in the environment. Nat Rev Microbiol.; 20(5):257-269. doi: 10.1038/s41579-021-00649-x.
12. Lamba, M., Graham, D.W. & Ahammad. S.Z. (2017). Hospital Wastewater Releases of Carbapenem-Resistance Pathogens and Genes in Urban India. Environmental Science & Technology 51 (23), 13906-13912. DOI: 10.1021/acs.est.7b03380
13. Marathe, N.P., Berglund, F., Razavi, M., Pal, C., Dröge, J., Samant, S., Kristiansson, E., Larsson, D.G.J. (2019). Sewage effluent from an Indian hospital harbor novel carbapenemases and integron-borne antibiotic resistance genes. Microbiome. 27;7(1):97. doi: 10.1186/s40168-019-0710-x
14. Praveenkumarreddy Y., Akiba M., Guruge K.S., Balakrishna K., Vandana K.E., Kumar V. (2020) Occurrence of antimicrobial-resistant escherichia coli in sewage treatment plants of south India. Journal of Water Sanitation and Hygiene for Development, 10 (1), pp. 48-55.
15. Talat, M., Khan, M. A., Ansari, M. I., Rehman, A., Usman, M., & Ahmad, I. (2023). Comprehensive examination of antibiotic resistomes in wastewater samples from six hospitals in various rural and urban regions of northern India using shotgun metagenomics. Journal of Environmental Science and Health, Part A, 58(5), 462-475.
16. Skariyachan, S., Mahajanakatti, A. B., Krishnanand, K., Krishna Prasad, M., Shenoy Mulky, V., & Ankathi, P. (2015). High prevalence of blaTEM mediated third-generation cephalosporin resistance in Escherichia coli isolates from the River Cauvery, India. Journal of Environmental Health Science and Engineering, 13, 13.
17. Azam, M., Jan, A. T., Haq, Q. M. R., Kamal, M. A., & Qadri, I. (2016). Prevalence of extended-spectrum β-lactamase (ESBL) producers among gram-negative bacteria isolated from the Yamuna River, Delhi, India. Frontiers in Microbiology, 7, 203.

18. Azam, M., Jan, A. T., & Haq, Q. M. (2016). blaCTX-M-152, a novel variant of CTX-M group-25, identified in a study performed on the prevalence of multidrug resistance among natural inhabitants of River Yamuna, India. Frontiers in Microbiology, 7, 1495. doi: 10.3389/fmicb.2016.01495
19. Devarajan, N., Laffite, A., Mulaji, C. K., Otamonga, J. P., Mpiana, P. T., Mubedi, J. I., ... Poté, J. (2016). Occurrence of antibiotic resistance genes and bacterial markers in a tropical river receiving hospital and urban wastewaters. PloS One, 11(2), e0149211. doi: 10.1371/journal.pone.0149211
20. Ahmad, A., Khan, M. U., & Balkrishnan, R. (2016). Fixed-dose combination antibiotics in India: Global perspectives. The Lancet Global Health, 4(8), e521-e522. doi: 10.1016/S2214-109X(16)30150-1
21. Marathe, N. P., Shetty, S. A., Shouche, Y. S., & Larsson, D. G. J. (2017). Untreated urban waste contaminates Indian river sediments with resistance genes to last resort antibiotics. Water Research, 124, 388-397. doi: 10.1016/j.watres.2017.07.065
22. Kumar, S., Mishra, A., Dey, D., Das, B., & Das, P. (2021). Prevalence of ampicillin and erythromycin resistance in bacterial isolates from municipal sewage water joining river Ganga, Prayagraj, India. Environmental Monitoring and Assessment, 193(7), 432.
23. Singh, A. K., Chaudhary, A., Kumar, A., Kumar, A., Sharma, A., & Singh, V. K. (2021). High prevalence of drug resistance and detection of class 1 integron gene intI in Escherichia coli strains isolated from river Yamuna, India. Environmental Science and Pollution Research, 28(39), 54710-54718.
24. Acharya, M., Ashworth, A. J., Yang, Y., Burke, J. M., Lee, J. A., & Sharma Acharya, R. (2021). Soil microbial diversity in organic and non-organic pasture systems. PeerJ, 9, e11184. doi:10.7717/peerj.11184.
25. Liguori, K., Keenum, I., Davis, B. C., Calarco, J., Milligan, E., Harwood, V. J., & Pruden, A. (2022). Antimicrobial Resistance Monitoring of Water Environments: A Framework for Standardized Methods and Quality Control. Environmental Science & Technology, 56(13), 9149-9160. doi: 10.1021/acs.est.1c08918.
26. Kaprou, G. D., Bergšpica, I., Alexa, E. A., Alvarez-Ordóñez, A., & Prieto, M. (2021). Rapid Methods for Antimicrobial Resistance Diagnostics. Antibiotics, 10(2), 209. doi: 10.3390/antibiotics10020209

27. Khameneh, B., Eskin, N. A. M., Iranshahy, M., & Fazly Bazzaz, B. S. (2021). Phytochemicals: A Promising Weapon in the Arsenal against Antibiotic-Resistant Bacteria. Antibiotics, 10(9), 1044. doi: 10.3390/antibiotics10091044.
28. Vaou, N., Stavropoulou, E., Voidarou, C., Tsigalou, C., & Bezirtzoglou, E. (2021). Towards Advances in Medicinal Plant Antimicrobial Activity: A Review Study on Challenges and Future Perspectives. Microorganisms, 9(10), 2041. doi: 10.3390/microorganisms9102041
29. Caudai, C., Galizia, A., Geraci, F., Le Pera, L., Morea, V., Salerno, E., Via, A., & Colombo, T. (2021). AI applications in functional genomics. Computational and Structural Biotechnology Journal, 19, 5762-5790. doi: 10.1016/j.csbj.2021.10.009. PMID: 34765093; PMCID: PMC8566780.
30. Pinzi, L., & Rastelli, G. (2019). Molecular Docking: Shifting Paradigms in Drug Discovery. International Journal of Molecular Sciences, 20(18), 4331. doi: 10.3390/ijms20184331.
31. Pinzi, L., & Rastelli, G. (2019). Molecular Docking: Shifting Paradigms in Drug Discovery. International Journal of Molecular Sciences, 20(18), 4331. doi: 10.3390/ijms20184331
32. Ehrlich, P. (1910). Anwendung und Wirkung von Salvarsan. Deutsche Medizinische Wochenschrift, 36(48), 2437-2438.
33. Hutchings, M. I., Truman, A. W., & Wilkinson, B. (2019). Antibiotics: past, present and future. Current Opinion in Microbiology, 51, 72-80. doi: 10.1016/j.mib.2019.10.008
34. Terreni, M., Taccani, M., & Pregnolato, M. (2021). New Antibiotics for Multidrug-Resistant Bacterial Strains: Latest Research Developments and Future Perspectives. Molecules, 26(9), 2671. doi: 10.3390/molecules26092671.
35. Reygaert, W. C. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. AIMS Microbiology, 4(3), 482-501. doi: 10.3934/microbiol.2018.3.482
36. Thursby, E., & Juge, N. (2017). Introduction to the human gut microbiota. Biochemical Journal, 474(11), 1823-1836. doi: 10.1042/BCJ20160510
37. Ramirez, J., Guarner, F., Bustos Fernandez, L., Maruy, A., Sdepanian, V. L., & Cohen, H. (2020). Antibiotics as Major Disruptors of Gut Microbiota. Frontiers in Cellular and Infection Microbiology, 10, 572912. doi: 10.3389/fcimb.2020.572912

38. Manyi-Loh, C., Mamphweli, S., Meyer, E., & Okoh, A. (2018). Antibiotic Use in Agriculture and Its Consequential Resistance in Environmental Sources: Potential Public Health Implications. Molecules, 23(4), 795. doi: 10.3390/molecules23040795
39. Aggarwal, M., Chakrabarti, A. S., Chatterjee, C., & Higgins, M. J. (2023). Research and market structure: Evidence from an antibiotic-resistant pathogenic outbreak. Research Policy, 52(1), 104633. ISSN 0048-7333. doi: 10.1016/j.respol.2022.104633.
40. Kim, D. W., & Cha, C. J. (2021). Antibiotic resistome from the One-Health perspective: understanding and controlling antimicrobial resistance transmission. Experimental & Molecular Medicine, 53(3), 301-309. doi: 10.1038/s12276-021-00569-z
41. Kunhikannan, S., Thomas, C. J., Franks, A. E., Mahadevaiah, S., Kumar, S., & Petrovski, S. (2021). Environmental hotspots for antibiotic resistance genes. Microbiologyopen, 10(3), e1197. doi: 10.1002/mbo3.1197.
42. Duan, Y., Chen, Z., Tan, L., Wang, X., Xue, Y., Wang, S., Wang, Q., Das, R., Lin, H., Hou, J., Li, L., Mao, D., & Luo, Y. (2020). Gut resistomes, microbiota and antibiotic residues in Chinese patients undergoing antibiotic administration and healthy individuals. Science of the Total Environment, 705, 135674. doi: 10.1016/j.scitotenv.2019.135674
43. Feng, J., Li, B., Jiang, X., Yang, Y., Wells, G. F., Zhang, T., & Li, X. (2017). Antibiotic resistome in a large-scale healthy human gut microbiota deciphered by metagenomic and network analyses. Environmental Microbiology, 20(1), 355-368. doi: 10.1111/1462-2920.14009
44. Watts, J. E. M., Schreier, H. J., Lanska, L., & Hale, M. S. (2017). The rising tide of antimicrobial resistance in aquaculture: sources, sinks and solutions. Marine Drugs, 15(6), 158. doi: 10.3390/md15060158
45. Xiong, W., Wang, Y., Sun, Y., Ma, L., Zeng, Q., Jiang, X., Li, A., Zeng, Z., & Zhang, T. (2018). Antibiotic-mediated changes in the fecal microbiome of broiler chickens define the incidence of antibiotic resistance genes. Microbiome, 6(1), 34. doi: 10.1186/s40168-018-0419-2
46. Dong, P., Wang, H., Fang, T., Wang, Y., & Ye, Q. (2019). Assessment of extracellular antibiotic resistance genes (eARGs) in typical environmental

samples and the transforming ability of eARG. Environment International, 125, 90-96. doi: 10.1016/j.envint.2019.01.050

47. Hendriksen, R. S., Munk, P., Njage, P., van Bunnik, B., McNally, L., Lukjancenko, O., Roder, T., Nieuwenhuijse, D., Pedersen, S. K., Kjeldgaard, J., Kaas, R. S., Clausen, P. T. L. C., Vogt, J. K., Leekitcharoenphon, P., van de Schans, M. G. M., Zuidema, T., de Roda Husman, A. M., Rasmussen, S., Petersen, B., Amid, C., Cochrane, G., Sicheritz-Ponten, T., Schmitt, H., Alvarez, J. R. M., Aidara-Kane, A., Pamp, S. J., Lund, O., Hald, T., Woolhouse, M., Koopmans, M. P., Vigre, H., Petersen, T. N., & Aarestrup, F. M. (2019). Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. Nature Communications, 10, 1124. doi: 10.1038/s41467-019-08853-3
48. Karkman, A., Do, T. T., Walsh, F., & Virta, M. P. J. (2018). Antibiotic-resistance genes in wastewater. Trends in Microbiology, 26(3), 220-228. doi: 10.1016/j.tim.2017.09.005
49. Wu, J., Su, Y., Deng, Y., Guo, Z., Cheng, C., Ma, H., Liu, G., Xu, L., & Feng, J. (2019). Spatial and temporal variation of antibiotic resistance in a marine fish cage-culture area of Guangdong, China. Environmental Pollution, 246, 463-471. doi: 10.1016/j.envpol.2018.12.024
50. Chen, Q., An, X., Li, H., Su, J., Ma, Y., & Zhu, Y.-G. (2016). Long-term field application of sewage sludge increases the abundance of antibiotic resistance genes in soil. Environmental International, 92-93, 1-10. doi: 10.1016/j.envint.2016.03.026
51. Han, X. M., Hu, H. W., Shi, X. Z., Wang, J. T., Han, L. L., Chen, D., & He, J. Z. (2016). Impacts of reclaimed water irrigation on soil antibiotic resistome in urban parks of Victoria, Australia. Environmental Pollution, 211, 48-57. doi: 10.1016/j.envpol.2015.12.033
52. Wu, N., Zhang, W., Xie, S., Zeng, M., Liu, H., Yang, J., Liu, X., & Yang, F. (2020). Increasing prevalence of antibiotic resistance genes in manured agricultural soils in northern China. Frontiers of Environmental Science & Engineering, 14(1), 1. doi: 10.1007/s11783-019-1180-x
53. Brągoszewska, E., & Biedron´, I. (2018). Indoor air quality and potential health risk impacts of exposure to antibiotic resistant bacteria in office rooms in

Southern Poland. International Journal of Environmental Research and Public Health, 15, 2604. https://doi.org/10.3390/ijerph15112604

54. Pal, C., Bengtsson-Palme, J., Kristiansson, E., & Larsson, D. G. J. (2016). The structure and diversity of human, animal and environmental resistomes. Microbiome, 4, 54. https://doi.org/10.1186/s40168-016-0199-5
55. Zhang, T., Li, X., Wang, M., Chen, H., Yang, Y., Chen, Q., & Yao, M. (2019). Time-resolved spread of antibiotic resistance genes in highly polluted air. Environment International, 127, 333–339. https://doi.org/10.1016/j.envint.2019.03.006
56. Zhuang, M., Achmon, Y., Cao, Y., Liang, X., Chen, L., Wang, H., Siame, B. A., & Leung, K. Y. (2021). Distribution of antibiotic resistance genes in the environment. Environmental Pollution, 285, 117402. ISSN 0269-7491. doi: 10.1016/j.envpol.2021.117402.
57. Delgado-Baquerizo, M., Hu, H. W., Maestre, F. T., Escolar, C., Abades, S., Goberna, M. & Singh, B. K. (2022). The global distribution and environmental drivers of the soil antibiotic resistome. Microbiome, 10, 219. doi: 10.1186/s40168-022-01405-w.
58. Wang, J., Pan, R., Dong, P., Lu, J., Jin, L., Wang, H. & Zhang, X. (2022). Supercarriers of antibiotic resistome in a world's large river. Microbiome, 10, 111. doi: 10.1186/s40168-022-01294-z.
59. Osińska, A., Korzeniewska, E., Harnisz, M., & Niestępski, S. (2017). The prevalence and characterization of antibiotic-resistant and virulent Escherichia coli strains in the municipal wastewater system and their environmental fate. Science of the Total Environment, 577, 367-375. doi: 10.1016/j.scitotenv.2016.10.203.
60. Van Gompel, L., Dohmen, W., Luiken, R., Bouwknegt, M., Heres, L., Heijnsbergen, E., Jongerius-Gortemaker, B.G.M., Scherpenisse, P., Greve, G., Tersteeg-Zijderveld, M., Wadepohl, K., Duarte, A.S., Muñoz-Gómez, V., Fischer, J., Skarżyńska, M., Wasyl, D., Wagenaar, J., Urlings, B., Dorado-García, A., Smit, L. (2019). Occupational Exposure and Carriage of Antimicrobial Resistance Genes (tetW, ermB) in Pig Slaughterhouse Workers. Annals of Work Exposures and Health, 63(5), 504-514. https://doi.org/10.1093/annweh/wxz098

61. Brinkac, L., Voorhies, A., Gomez, A., & Nelson, K. E. (2017). The threat of antimicrobial resistance on the human microbiome. Microbial Ecology, 74(4), 1001-1008. https://doi.org/10.1007/s00248-017-0985-z
62. Kapoor, G., Saigal, S., & Elongavan, A. (2017). Action and resistance mechanisms of antibiotics: A guide for clinicians. Journal of Anaesthesiology Clinical Pharmacology, 33(3), 300-305. https://doi.org/10.4103/joacp.JOACP_349_15
63. World Health Organization. (2017). WHO publishes list of bacteria for which new antibiotics are urgently needed. [Press release]. Retrieved from https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed
64. Mueller, M., & Tainter, C. R. (2023). Escherichia Coli. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing. PMID: 33231968.
65. Annual Report, AMR surveillance Network, Indian Council of Medical Research (2021).
66. Khan, A. U., Maryam, L., & Zarrilli, R. (2017). Structure, genetics and worldwide spread of New Delhi metallo-β-lactamase (NDM): A threat to public health. BMC Microbiology, 17, 101. https://doi.org/10.1186/s12866-017-1012-8
67. Fils, P. E. L., Cholley, P., Gbaguidi-Haore, H., Hocquet, D., Sauget, M., Bertrand, X. (2021). ESBL-producing Klebsiella pneumoniae in a university hospital: Molecular features, diffusion of epidemic clones and evaluation of cross-transmission. PLoS ONE, 16(3), e0247875. https://doi.org/10.1371/journal.pone.0247875
68. Kaprou, G. D., Bergšpica, I., Alexa, E. A., Alvarez-Ordóñez, A., & Prieto, M. (2021). Rapid methods for antimicrobial resistance diagnostics. Antibiotics, 10(2), 209. https://doi.org/10.3390/antibiotics10020209
69. Vasala, A., Hytönen, V. P., & Laitinen, O. H. (2020). Modern tools for rapid diagnostics of antimicrobial resistance. Frontiers in Cellular and Infection Microbiology, 10, 337. https://doi.org/10.3389/fcimb.2020.00337
70. Liu, J., Zhang, Q., Dong, Y. Q., Yin, J., & Qiu, Y. Q. (2022). Diagnostic accuracy of metagenomic next-generation sequencing in diagnosing infectious diseases: a meta-analysis. Scientific Reports, 12(1), 21032. doi: 10.1038/s41598-022-25314-y. PMID: 36470909; PMCID: PMC9723114.

71. Navgire, G. S., Goel, N., Sawhney, G., Gupta, S., Saraf, M., & Bharti, S. (2022). Analysis and Interpretation of metagenomics data: an approach. Biology Procedures Online, 24, 18. doi: 10.1186/s12575-022-00179-7.
72. Prayogo, F. A., Budiharjo, A., Kusumaningrum, H. P., Wijanarka, W., Suprihadi, A., & Nurhayati, N. (2020). Metagenomic applications in exploration and development of novel enzymes from nature: a review. Journal of Genetic Engineering and Biotechnology, 18(1), 39. doi: 10.1186/s43141-020-00043-9. PMID: 32749574; PMCID: PMC7403272.
73. Gupta, N., & Verma, V. K. (2019). Next-Generation Sequencing and Its Application: Empowering in Public Health Beyond Reality. In Microbial Technology for the Welfare of Society (pp. 313-341). doi: 10.1007/978-981-13-8844-6_15. PMCID: PMC7122948.
74. Zhang, L., Chen, F. X., Zeng, Z., Xu, M., Sun, F., Yang, L., Bi, X., Lin, Y., Gao, Y., Hao, H., Yi, W., Li, M., & Xie, Y. (2021). Advances in Metagenomics and Its Application in Environmental Microorganisms. Frontiers in Microbiology, 12, 766364. doi: 10.3389/fmicb.2021.766364. ISSN: 1664-302X.
75. Ngara, T. R., & Zhang, H. (2018). Recent Advances in Function-based Metagenomic Screening. Genomics, Proteomics & Bioinformatics, 16(6), 405-415. doi: 10.1016/j.gpb.2018.01.002.
76. Franklin, A. M., Brinkman, N. E., Jahne, M. A., & Keely, S. P. (2021). Twenty-first century molecular methods for analyzing antimicrobial resistance in surface waters to support One Health assessments. Journal of Microbiological Methods, 184, 106174. doi: 10.1016/j.mimet.2021.106174. PMID: 33774111; PMCID: PMC8159016.
77. de Abreu, V. A. C., Perdigão, J., & Almeida, S. (2021). Metagenomic Approaches to Analyze Antimicrobial Resistance: An Overview. Frontiers in Genetics, 11, 575592. doi: 10.3389/fgene.2020.575592. PMID: 33537056; PMCID: PMC7848172.
78. Kanehisa, M., Goto, S., Kawashima, S., Okuno, Y., & Hattori, M. (2004). The KEGG resource for deciphering the genome. Nucleic Acids Research, 32(Database issue), D277-D280. https://doi.org/10.1093/nar/gkh063
79. Muller, J., Szklarczyk, D., Julien, P., Letunic, I., Roth, A., Kuhn, M., Powell, S., von Mering, C., Doerks, T., Jensen, L. J., Bork, P. (2010). eggNOG v2.0:

Extending the evolutionary genealogy of genes with enhanced non-supervised orthologous groups, species and functional annotations. Nucleic Acids Research, 38(Database issue), D190-D195. https://doi.org/10.1093/nar/gkp951

80. Tatusov, R. L., Fedorova, N. D., Jackson, J. D., Jacobs, A. R., Kiryutin, B., Koonin, E. V., Krylov, D. M., Mazumder, R., Mekhedov, S. L., Nikolskaya, A. N., Rao, B. S., Smirnov, S., Sverdlov, A. V., Vasudevan, S., Wolf, Y. I., Yin, J. J., Natale, D. A. (2003). The COG database: An updated version includes eukaryotes. BMC Bioinformatics, 4, 41. https://doi.org/10.1186/1471-2105-4-41
81. Finn, R.D., Mistry, J., Tate, J., Coggill, P., Heger, A., Pollington, J.E., Gavin, O.L., Gunasekaran, P., Ceric, G., Forslund, K., Holm, L., Sonnhammer, E.L., Eddy, S.R., Bateman, A. (2010). The Pfam protein families database. Nucleic Acids Research, 38(Database issue), D211-D222.
82. Selengut, J.D., Haft, D.H., Davidsen, T., Ganapathy, A., Gwinn-Giglio, M., Nelson, W.C., Richter, A.R., White, O. (2007). TIGRFAMs and Genome Properties: Tools for the assignment of molecular function and biological process in prokaryotic genomes. Nucleic Acids Research, 35(Database issue), D260-D264.
83. Gupta, C. L., Tiwari, R. K., & Cytryn, E. (2020). Platforms for elucidating antibiotic resistance in single genomes and complex metagenomes. Environment International, 138, 105667. doi: 10.1016/j.envint.2020.105667.
84. Pillay, S., San, J. E., Tshiabuila, D., Naidoo, Y., Pillay, Y., Maharaj, A., Anyaneji, U. J., Wilkinson, E., Tegally, H., Lessells, R. J., Baxter, C., de Oliveira, T., & Giandhari, J. (2023). Evaluation of miniaturized Illumina DNA preparation protocols for SARS-CoV-2 whole genome sequencing. PLoS One, 18(4), e0283219. doi: 10.1371/journal.pone.0283219.
85. Cave, R., Cole, J., & Mkrtchyan, H. V. (2021). Surveillance and prevalence of antimicrobial resistant bacteria from public settings within urban built environments: Challenges and opportunities for hygiene and infection control. Environmental International, 157, 106836. doi: 10.1016/j.envint.2021.106836.
86. Fu, Y., Zhao, J., & Chen, Z. (2018). Insights into the Molecular Mechanisms of Protein-Ligand Interactions by Molecular Docking and Molecular Dynamics Simulation: A Case of Oligopeptide Binding Protein. Computational and

Mathematical Methods in Medicine, 2018, 3502514. doi: 10.1155/2018/3502514.

87. Monod, J., Wyman, J., & Changeux, J. P. (1965). On the nature of allosteric transitions: A plausible model. Journal of Molecular Biology, 12(1), 88-118.
88. Frauenfelder, H., Sligar, S. G., & Wolynes, P. G. (1991). The energy landscapes and motions of proteins. Science, 254(5038), 1598-1603.
89. Austin, R. H., Beeson, K. W., Eisenstein, L., Frauenfelder, H., & Gunsalus, I. C. (1975). Dynamics of ligand binding to myoglobin. Biochemistry, 14(23), 5355-5373.
90. Foote, J., & Milstein, C. (1994). Kinetic maturation of an antibody response. Nature, 352(6338), 530-532
91. Kumar, S., Ma, B., & Tsai, C. J. (2000). Sinha N, Nussinov R. Folding and binding cascades: Dynamic landscapes and population shifts. Protein Science, 9(1), 10-19.
92. Kobilka, B. K., & Deupi, X. (2007). Conformational complexity of G-protein-coupled receptors. Trends in Pharmacological Sciences, 28(8), 397-406.
93. Okazaki, K., & Takada, S. (2008). Dynamic energy landscape view of coupled binding and protein conformational change: Induced-fit versus population-shift mechanisms. Proceedings of the National Academy of Sciences, 105(32), 11182-11187.
94. Zhou, H. X. (2010). From induced fit to conformational selection: A continuum of binding mechanism controlled by the timescale of conformational transitions. Biophysical Journal, 98(6), L15-L17.
95. Csermely, P., Palotai, R., & Nussinov, R. (2010). Induced fit, conformational selection and independent dynamic segments: An extended view of binding events. Trends in Biochemical Sciences, 35(10), 539-546.
96. Torres, M. (2019). Drug discovery and development. In H. M. S. Gustavo and L. Y. S. Eliezer (Eds.), Comprehensive Medicinal Chemistry III (pp. 1-39). Elsevier.
97. Azad, A. (2023). Simplifying the docking process: Focusing on protein-ligand interaction. Journal of Molecular Modeling, 29(3), 74. doi: 10.1007/s00894-023-05423-4

98. Pinzi, L. (2019). Considerations on ligand binding conformations in molecular docking simulations. Drug Discovery Today, 24(5), 1154-1161. doi: 10.1016/j.drudis.2019.02.006.
99. Decherchi, S. (2020). Residence Time Analysis in Drug Discovery: Past, Present, and Future. Journal of Medicinal Chemistry, 63(7), 3503-3522. doi: 10.1021/acs.jmedchem.9b01811.
100. Salo-Ahen, O. M. H. (2020). Homology Modeling in Structure-Based Drug Discovery. Methods in Molecular Biology, 2127, 1-12. doi: 10.1007/978-1-0716-0361-1_1.
101. Pellicani, F., Dal Ben, D., Perali, A., & Pilati, S. (2023). Machine Learning Scoring Functions for Drug Discovery from Experimental and Computer-Generated Protein–Ligand Structures: Towards Per-Target Scoring Functions. Molecules, 28(4), 1661. https://doi.org/10.3390/molecules28041661
102. Guedes, I. A., Pereira, F. S. S., & Dardenne, L. E. (2018). Empirical Scoring Functions for Structure-Based Virtual Screening: Applications, Critical Aspects, and Challenges. Frontiers in Pharmacology, 9, 1089. doi: 10.3389/fphar.2018.01089
103. Sarecka-Hujar, B., & Szulc-Musioł, B. (2022). Herbal Medicines-Are They Effective and Safe during Pregnancy? Pharmaceutics, 14(1), 171. doi: 10.3390/pharmaceutics14010171.
104. Jubair, N., Rajagopal, M., Chinnappan, S., Abdullah, N. B., & Fatima, A. (2021). Review on the Antibacterial Mechanism of Plant-Derived Compounds against Multidrug-Resistant Bacteria (MDR). Evid Based Complement Alternat Med, 2021, 3663315. doi: 10.1155/2021/3663315.
105. Charles, N., Adelani-Akande, T., Osemwegie, O., Olaniran, A., & Adeyemo, T. (2020). Preliminary in vitro antimicrobial potential and phytochemicals study of some medical plants. F1000Research, 8, 81. doi: 10.12688/f1000research.17094.3.
106. Murugan Prasathkumar, Salim, A., Chenthamara, D., Robert, B., & Subramaniam, S. (2021). Therapeutic and pharmacological efficacy of selective Indian medicinal plants – A review. Phytomedicine Plus, 1(2), 100029. https://doi.org/10.1016/j.phyplu.2021.100029
107. Hazra, S., Ray, A. S., Das, S., Das Gupta, A., & Rahaman, C. H. (2023). Phytochemical Profiling, Biological Activities, and In Silico Molecular

Docking Studies of Causonis trifolia (L.) Mabb. & J.Wen Shoot. Plants, 12(7), 1495. https://doi.org/10.3390/plants12071495

108. Pott, D. M., Osorio, S., & Vallarino, J. G. (2019). From Central to Specialized Metabolism: An Overview of Some Secondary Compounds Derived From the Primary Metabolism for Their Role in Conferring Nutritional and Organoleptic Characteristics to Fruit. Frontiers in Plant Science, 10, 835. doi: 10.3389/fpls.2019.00835. PMID: 31316537; PMCID: PMC6609884.
109. Eljounaidi, K., & Lichman, B. R. (2020). Nature's Chemists: The Discovery and Engineering of Phytochemical Biosynthesis. Frontiers in Chemistry, 8, 596479. doi: 10.3389/fchem.2020.596479. PMID: 33240856; PMCID: PMC7680914.
110. Cheesman, M. J. (2017). Plants and plant extracts for improving microbiological safety of poultry products. In D. K. Atungulu (Ed.), Poultry Quality Evaluation: Quality Attributes and Consumer Values (pp. 71-92). Academic Press.
111. Vaou, M. (2021). Phytochemicals as alternative antimicrobial agents in the era of antibiotic resistance: A review. Pharmaceuticals, 14(4), 366. doi: 10.3390/ph14040366.
112. Roy, R. (2018). Plant-derived antimicrobials: A potential solution to antibiotic resistance? Antibiotics, 7(2), 51. doi: 10.3390/antibiotics7020051.
113. Sharma, A. (2019). Plant-derived antimicrobials as potential inhibitors of efflux pumps in drug-resistant bacteria: Current status and future perspectives. Frontiers in Microbiology, 10, 1997. doi: 10.3389/fmicb.2019.01997.
114. Tako, E. A. (2020). Plant-derived antimicrobials as potential inhibitors of bacterial virulence factors: A promising approach to enhance antibiotic efficacy. Frontiers in Microbiology, 11, 187. doi: 10.3389/fmicb.2020.00187.
115. Mahato, S. B. (2019). Phytochemicals: Isolation, characterization, and role in drug discovery. In Medicinal Plants: Chemistry, Biology and Omics (pp. 297-336). Elsevier.
116. Miethke, M. (2021). Combining antibiotics and plant compounds for enhanced antimicrobial activity against multidrug-resistant bacteria. Frontiers in Microbiology, 12, 706664. doi: 10.3389/fmicb.2021.706664
117. Nayak, S. (2013). Adaptations of pteridophytes to shade conditions: A review. Indian Fern Journal, 30, 112-120.

118. Nath, S. C. (2017). Therapeutic potential of ferns. Journal of Ayurveda and Integrative Medicine, 8(4), 253-259. doi: 10.1016/j.jaim.2017.04.001.
119. Mandal, M. K. (2020). Ferns: Nature's pharmaceutical factory. Journal of Ethnopharmacology, 254, 112682. doi: 10.1016/j.jep.2020.112682.
120. Banerjee, S., & Sen, S. (1980). Antibiotic activity in some common ferns. Current Science, 49(5), 187-188.
121. Kumar, R., Sood, S., Sharma, S., Sharma, S., & Vyas, A. (2003). Ethnobotanical and phytochemical studies of Christella dentata (Forssk.) & Jermya. Journal of Ethnopharmacology, 84(2-3), 199-204.
122. Kumar, A., & Dash, S. K. (2012). Ethnomedicinal uses and pharmacological activities of ferns of Odisha, India: A review. Journal of Medicinal Plants Research, 6(33), 4842-4855.
123. Singh, M., Ganguli, S., & Ghosh, M. (2020). Antimicrobial and computational assessment of Christella dentata crude extracts against multidrug-resistant bacterial cultures and targets. Journal of Environment and Sociobiology (pp. 21-22).
124. Linton, A. H., Howe, K., & Bennett, P. M. (1974). Antimicrobial resistance in hospital and urban wastewater. The Lancet, 2(7887), 1316-1318.
125. CLSI guidelines: 2019. Clinical and Laboratory Standards Institute Standards Development Policies and Process.
126. Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W. & Higgins, D. G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Molecular systems biology, 7(1), 539.
127. Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic acids research, 32(5), 1792-1797.
128. Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular biology and evolution, 4(4), 406-425.
129. Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. Journal of molecular evolution, 17(6), 368-376.
130. Ganguli, S., Pahwa, S., Sahu, A., Kumar, V., & Kumar, R. (2011). MOLINSPIRATION: A New Machine Learning-Based Drug Discovery Approach. Journal of Chemical Information and Modeling, 51(11), 2900-2907.

131. Salma, U., Hussain, W., & Hussain, J. (2017). MOLINSPIRATION v2.0: A Platform for in silico Drug Discovery. Journal of Cheminformatics, 9(1), 39.
132. Duhovny, D., Nussinov, R., & Wolfson, H. J. (2005). Efficient unbound docking of rigid molecules. In Proceedings of the 1st ACM International Conference on Bioinformatics, Computational Biology and Biomedicine (pp. 185-194).
133. Wallace, A. C., Laskowski, R. A., & Thornton, J. M. (1995). LIGPLOT: A program to generate schematic diagrams of protein-ligand interactions. Protein Engineering, Design and Selection, 8(2), 127-134. doi: 10.1093/protein/8.2.127.
134. Lupo, A., Coyne, S., Berendonk, T.U. (2012). Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies. Frontiers in Microbiology, 3, 18.
135. Gullberg, E., Cao, S., Berg, O.G., Ilbäck, C., Sandegren, L., Hughes, D., et al. (2011). Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathogens, 7(7), e1002158.
136. Martinez, J.L., Sanchez, M.B., Martinez-Solano, L., Hernandez, A., Garmendia, L., Fajardo, A., et al. (2009). Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. FEMS Microbiology Reviews, 33, 430-449.
137. Lipinski, C. A., Lombardo, F., Dominy, B. W., & Feeney, P. J. (2001). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Advanced Drug Delivery Reviews, 46(1-3), 3-26. doi: 10.1016/s0169-409x(00)00129-0.
138. Santos-Lopez, A., Marshall, C. W., Scribner, M. R., Snyder, D. J., & Cooper, V. S. (2019). Evolutionary pathways to antibiotic resistance are dependent upon environmental structure and bacterial lifestyle. eLife, 8, e47612. https://doi.org/10.7554/eLife.47612
139. Schmiege, D., Zacharias, N., Sib, E., Falkenberg, T., Moebus, S., Evers, M., & Kistemann, T. (2021). Prevalence of multidrug-resistant and extended-spectrum beta-lactamase-producing Escherichia coli in urban community wastewater. Science of the Total Environment, 785, 147269. https://doi.org/10.1016/j.scitotenv.2021.147269

140. Feldman, S. F., Temkin, E., Wullfhart, L., Nutman, A., Schechner, V., Shitrit, P., Shvartz, R., Schwaber, M. J., Andremont, A., & Carmeli, Y. (2022). A nationwide population-based study of Escherichia coli bloodstream infections: incidence, antimicrobial resistance and mortality. Clinical Microbiology and Infection, 28(6), 879.e1-879.e7. https://doi.org/10.1016/j.cmi.2021.12.009

141. Shibuki, R., Nishiyama, M., Mori, M., Baba, H., Kanamori, H., & Watanabe, T. (2023). Characterization of extended-spectrum β-lactamase-producing Escherichia coli isolated from municipal and hospital wastewater in Japan. Journal of Global Antimicrobial Resistance, 32, 145-151. https://doi.org/10.1016/j.jgar.2023.02.002

142. Caneiras, C., Lito, L., Melo-Cristino, J., & Duarte, A. (2019). Community- and Hospital-Acquired Klebsiella pneumoniae Urinary Tract Infections in Portugal: Virulence and Antibiotic Resistance. Microorganisms, 7(5), 138. https://doi.org/10.3390/microorganisms7050138

143. Anjum, M. F., Schmitt, H., Börjesson, S., Berendonk, T. U., Donner, E., Stehling, E. G., Boerlin, P., Topp, E., Jardine, C., Li, X., Li, B., Dolejska, M., Madec, J.-Y., Dagot, C., Guenther, S., Walsh, F., Villa, L., Veldman, K., Sunde, M., Krzeminski, P., Wasyl, D., Popowska, M., Järhult, J., Örn, S., Mahjoub, O., Mansour, W., Thái, Đ. N., Elving, J., & Pedersen, K. (2021). The potential of using E. coli as an indicator for the surveillance of antimicrobial resistance (AMR) in the environment. Current Opinion in Microbiology, 64, 152-158. https://doi.org/10.1016/j.mib.2021.09.011

144. Castanheira, M., Simner, P. J., Bradford, P. A. (2021). Extended-spectrum β-lactamases: an update on their characteristics, epidemiology and detection. Journal of Antimicrobial Chemotherapy, 76(4), 857-868.

145. Cheng, V. C. C., Wong, S. C., Wong, S. C. Y., Ho, P. L., Yuen, K. Y. (2018). Control of carbapenemase-producing enterobacteriaceae: beyond the hospital. EClinicalMedicine, 6, 3-4.

146. Harmon, D. E., Miranda, O. A., McCarley, A., Eshaghian, M., Carlson, N., Ruiz, C. (2019). Prevalence and characterization of carbapenem-resistant bacteria in water bodies in the Los Angeles-Southern California area. MicrobiologyOpen, 8(4), e00692.

147. Klein, E. Y., van Boeckel, T. P., Martinez, E. M., Pant, S., Gandra, S., Levin, S. A., Goossens, H., Laxminarayan, R. (2018). Global increase and geographic

convergence in antibiotic consumption between 2000 and 2015. Proceedings of the National Academy of Sciences, 115(15), E3463-E3470.

148. Rocha, J., Ferreira, C., Mil-Homens, D., Lopes, A., Correia, A., Pinto, L. & Duarte, A. (2022). Third generation cephalosporin-resistant Klebsiella pneumoniae thriving in patients and in wastewater: what do they have in common? BMC Genomics, 23(1), 72. https://doi.org/10.1186/s12864-021-08279-6
149. Radisic, V., Grevskott, D. H., Lunestad, B. T., Øvreås, L., & Marathe, N. P. (2023). Sewage-based surveillance shows presence of Klebsiella pneumoniae resistant against last resort antibiotics in the population in Bergen, Norway. International Journal of Hygiene and Environmental Health, 248, 114075. https://doi.org/10.1016/j.ijheh.2022.114075
150. Puzari, M., Sharma, M., Chetia, P. (2018). Emergence of antibiotic resistant Shigella species: a matter of concern. Journal of Infection and Public Health, 11(4), 451-454.
151. Ma, Q., Huang, Y., Wang, J., Xu, X., Hawkey, P. M., Yang, C., ... Yang, X. (2018). Multidrug-resistant Shigella sonnei carrying the plasmid-mediated mcr-1 gene in China. International Journal of Antimicrobial Agents, 52(1), 14-21.
152. Ranjbar, R., & Farahani, A. (2019). Shigella: Antibiotic-Resistance Mechanisms And New Horizons For Treatment. Infection and Drug Resistance, 12, 3137-3167. doi: 10.2147/IDR.S219755. PMID: 31632102; PMCID: PMC6789722.
153. CDC. (2023). National Antimicrobial Resistance Monitoring System (NARMS) Now: Human Data. Atlanta, Georgia: U.S. Department of Health and Human Services, CDC.
154. Wang, J., et al. (2010). Isolation of a Lysinibacillus fusiformis strain with tetrodotoxin-producing ability from puffer fish Fugu obscurus and the characterization of this strain. Toxicon, 56(8), 1436-1441.
155. Taieb, I., Ben Younes, S., Messai, B., Mnif, S., Mzoughi, R., Bakhrouf, A., ... Ellafi, A. (2021). Isolation, Characterization and Identification of a New Lysinibacillus fusiformis Strain ZC from Metlaoui Phosphate Laundries Wastewater: Bio-Treatment Assays. Sustainability, 13(18), 10072.
156. Zhong, F., Wu, J., Dai, Y., Yang, L., Zhang, Z., Cheng, S., & Zhang, Q. (2015). Bacterial community analysis by PCR-DGGE and 454-pyrosequencing of

horizontal subsurface flow constructed wetlands with front aeration. Applied Microbiology and Biotechnology, 99(4), 1499-1512.

157. Handschuh, H., Ryan, M. P., O'Dwyer, J., Adley, C. C. (2017). Assessment of the bacterial diversity of aircraft water: Identification of the frequent fliers. PLoS ONE, 12(2), e0170567.
158. Xiong, J., Li, D., Li, H., He, M., Miller, S. J., Yu, L., ... Wang, G. (2011). Genome analysis and characterization of zinc efflux systems of a highly zinc-resistant bacterium, Comamonas testosteroni S44. Research in Microbiology, 162(7), 671-679.
159. Andrade, G., Esteban, E., Velasco, L., Lorite, M. J., Bedmar, E. J. (1997). Isolation and identification of N2-fixing microorganisms from the rhizosphere of Capparis spinosa (L.). Plant and Soil, 197(1), 19-23.
160. Pavone, S., Rinoldo, R., Albini, E., Fiorucci, A., Caponi, B., Fratto, A., ... Magistrali, C. F. (2021). First report of urinary tract infection caused by Comamonas kerstersii in a goat. BMC Veterinary Research, 17(1), 1-5.
161. Hem, S., Wyrsch, E. R., Drigo, B., Baker, D. J., Charles, I. G., Donner, E., ... Djordjevic, S. P. (2022). Genomic Analysis of Carbapenem-Resistant Comamonas in Water Matrices: Implications for Public Health and Wastewater Treatments. Applied and Environmental Microbiology, 88(1), e00646-22.
162. Jiang, X., Cui, X., Liu, W., Xu, H., & Zheng, B. (2019). Genetic characterization of a novel sequence type of multidrug-resistant Citrobacter freundii strain recovered from a wastewater treatment plant. Infection and Drug Resistance, 12, 2775-2779.
163. An, D. S., Im, W. T., Yang, H. C., & Lee, S. T. (2007). Terribacillus halophilus gen. nov., sp. nov., an extremely halophilic bacterium in the family Bacillaceae. Journal of Microbiology, 45(5), 515-521. PMID: 17978803.

APPENDIX

List of publications

1. Meesha Singh, Sayak Ganguli, Mahashweta Mitra Ghosh; “Comparative Metagenomic Dataset of hospital effluent microbiome from rural and urban hospitals in West Bengal” Data in Brief. 2019.
2. Meesha Singh, Sayak Ganguli, Mahashweta Mitra Ghosh; “Phytochemical, antimicrobial and computational assessment Of Christella dentata crude extracts against multidrug resistant bacterial cultures and targets”. J. Environ. & Sociobiol.: 17(1) : 13-19, 2020.
3. Subhoshmita Mondal, Sohini Gupta, Meesha Singh, Somosree Pal, Kaustav Das, Mahashweta Mitra Ghosh, Subrata Sankar Bagchi, Sayak Ganguli; “A Pipeline for Assessment of Pathogenic Load in the Environment Using Microbiome Analysis”. Springer, 2020.
4. Meesha Singh, Rupsha Karmakar, Sayak Ganguli, Mahashweta Mitra Ghosh; “Report of Antibiotic Resistance in Urban and Rural Wastewaters from West Bengal, India”, Journal of Pharmaceutical Research International, 33(53A), pp. 274-285, 2021.
5. Sayak Ganguli, Rupsha Karmakar, Meesha Singh, Mahashweta Mitra Ghosh. "Metagenomics-Guided Assessment of Water Quality and Predicting Pathogenic Load." In Handbook of Research on Monitoring and Evaluating the Ecological Health of Wetlands. edited by Rathoure, Ashok K., 71-91. Hershey, PA: IGI Global, 2022.

Poster & Oral Presentations

1. Poster Presentation entitled: “Biochemical and Antimicrobial Evaluation of crude extracts of Christella and Diplazium species collected from Lepchakha village (3500 ft) and computational studies of their active principles against targets from multidrug resistant E.coli, P aeruginosa, K pneumoniae and S aureus – common pathogens found in hospital effluents” at International Seminar on Recent Trends in Science towards sustainable development at APC College, Barrackpore 3rd and 4th August 2018

2. Poster Presentation entitled: “Antibacterial Screening of some native ferns against multidrug resistant organisms isolated from hospital effluents” at International Conference on Contemporary Antimicrobial Research (ICCAR) at IIT Kharagpur, 15th to 17th December 2018.
3. Poster Presentation entitled: “Characterization of bacterial isolates from hospital wastewater and antibiotic susceptibility assay to determine Multi-drug resistance patterns”. Frontiers in Biological Sciences (Chapter III), St. Xavier’s College, Kolkata (2019)

Data in brief 25 (2019) 104264

Contents lists available at ScienceDirect

Data in brief

journal homepage: www.elsevier.com/locate/dib

Data Article

Comparative metagenomic dataset of hospital effluent microbiome from rural and urban hospitals in West Bengal

Meesha Singh [a], Sayak Ganguli [b], Mahashweta Mitra Ghosh [a, *]

[a] Department of Microbiology, St. Xavier's College, Kolkata 700016, India
[b] Theoretical and Computational Biology Division, AIIST, Palta 742122, India

ARTICLE INFO

Article history:
Received 17 May 2019
Received in revised form 6 July 2019
Accepted 9 July 2019
Available online 17 July 2019

Keywords:
Illumina
Metagenomics data
Hospital wastewater
Antibiotic resistance

ABSTRACT

The unsafe disposal of hospital effluents contributes to gross contamination of water bodies with antibiotic residues, antibiotic resistance genes and antibiotic resistance bacteria. This study reports the microbial community profile of hospital wastes collected from various regions of West Bengal, India, using 16S rRNA gene amplicon sequencing. The data set Liquid Sludge (LS) contains 15,372,973 reads with an average length of 301 bps with average 52 ± 5% GC content. The data set Solid Sludge (SS) contains 16,071,594 reads with an average length of 301 bps with average 53 ± 4% GC content. Data of this study are available at NCBI BioProject (PRJNA360379). In sample LS, an abundance of 19.3% for the members of Bacteroidetes was observed. In sample SS, an abundance of 19.7% for the members of Euryarchaeota was observed.

1. Data

This data reports the occurrence of microbial abundance in hospital effluents collected from Purulia (LS) and Kolkata (SS), West Bengal, India.

* Corresponding author.,
E-mail addresses: mahasweta.mitra@sxccal.edu, mmgxaviers@gmail.com (M.M. Ghosh).

https://doi.org/10.1016/j.dib.2019.104264

Specifications table

Subject area	Microbiology
More specific subject area	Microbial Genomics
Type of data	NGS based Metagenomics data represented by Pie chart and Venn diagram
How data was acquired	Aseptic collection of hospital effluents from rural and urban areas
Data format	Raw FASTQ file
Experimental factors	16S rRNA gene amplicon sequencing
Experimental features	Raw sequence reads generated using Amplicon sequencing of V3–V4 regions
Data source location	Purulia and Kolkata, West Bengal, India
Data accessibility	https://www.ncbi.nlm.nih.gov/sra/PRJNA541056

Value of the data

- This data is the first report on the identification of microbial abundance and potential pathogenic strains from hospital effluents from an urban and rural multi-specialty hospital of West Bengal using metagenomics.
- The abundance profiles provide an insight on multiple parameters such as the presence or absence of potential pathogenic strains as well as the prevalence of disease associated microbes.
- Medical practitioners, common people and policy makers can benefit from the data in different ways. While the first group can understand the efficacy and threat of the overuse of specific antibiotics, the second group can take self-remedial measures during the visit to such public medical facilities. Finally, the policy makers should take note towards the implementation of effective treatment and mitigation strategies for such effluents.
- The data generated indicates the prevalence of resistant microbes in a rural environmental effluent as compared to an urban site indicative of the lack of awareness from a sociopolitical perspective, thus, indicating that stronger awareness measures need to be undertaken.
- The contamination of fishes reared in fresh water and waste water with antibiotic resistant bacteria has been reported in West Bengal which might be a fallout of these effluents finding their way into the riverine system and hence the data generated is an important parametric standard towards the understanding of the source of such potential bio-magnification events [1]. Further, this leads us to throw light on hospital associated spread of antibiotic resistance.

The data set LS contains 15,372,973 reads with an average length of 301 bps as shown in Fig. 1(a). Of the sequences that passed QC, 28,095 sequences (93%) contain ribosomal RNA genes, 1,104 sequences (3.65%) contain predicted proteins with known functions, and 1,069 sequences (3.53%) contain predicted proteins with unknown function.

The data set SS contains 16,071,594 reads with an average length of 301 bps represented by Fig. 1(a). Of the sequences that passed QC, 12,171 sequences (59%) contain ribosomal RNA genes, 1,885 sequences (9.21%) contain predicted proteins with known functions, and 6,403 sequences (31.30%) contain predicted proteins with unknown function.

In sample LS, the community study revealed an abundance of 19.3 % for the members of Bacteroidetes. In sample SS, the community study revealed an abundance of 19.7 % for the members of Euryarchaeota.

As shown in Fig. 1(b), at the genus level, *Prevotella* was the most dominant microbial member with abundance of 17.47% in LS. For SS, *Methanosaeta* was the dominant genus with abundance of 16.47%.

The comparative genus diversity LS and SS sample sets is represented by Fig. 1(c) with *Prevotella*, *Bacillus*, *Clostridium* etc. as the common genera.

2. Experimental design, materials and methods

The untreated hospital wastewater and sludge were aseptically collected in sterile containers from rural and urban regions of West Bengal from the main drainage systems of the hospitals aseptically. The effluents were transported within 10 hours of collection. The samples were stored at −20 °C until further processing.

The DNA isolation from each sample was done following the protocol by Bonet et al., 2012 [2]. The quantification of DNA was performed using Qubit dsDNA HS Assay kit (Life Tech). The concentration was determined by Qubit® Fluorometer by taking 1 μl of each sample. The microbial genomic DNA from hospital effluents was normalized to concentration <10 ng/μl. The PCR library preparation of amplicons

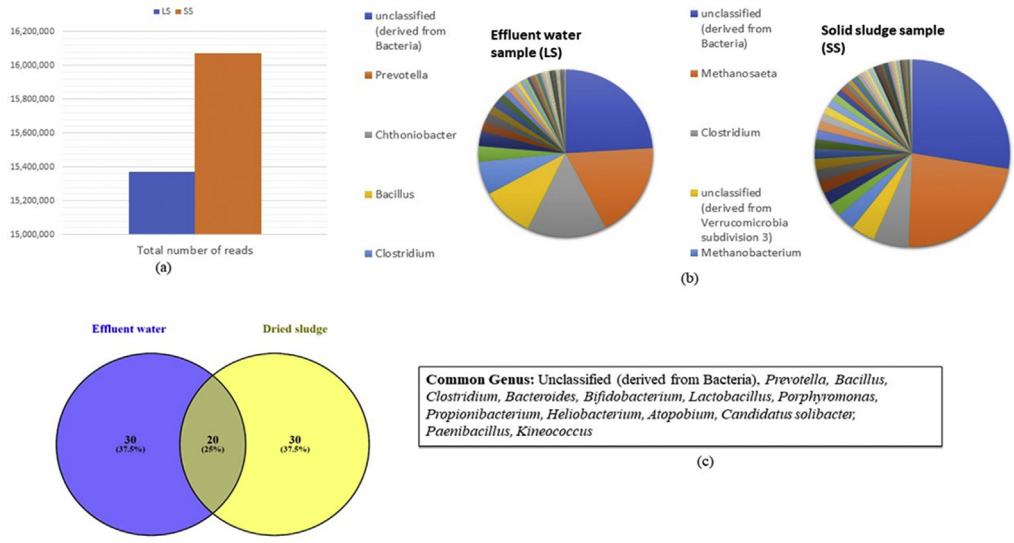


Fig. 1. Representation of analyzed data: (a): Histogram representing total number of reads for both LS and SS samples; (b): Pie charts representing genus diversity of the effluent metagenome LS and SS; (c): Venn diagram representing the common genus among the LS and SS samples.

was carried out using Nextera XT Idex Kit (Illumina, Inc.). The 16S Metagenomic Sequencing Library preparation protocol was followed. Primers for the amplification of the V3–V4 hyper-variable region [V3 Forward Oligo: CCTACGGGNBGCASCAG and V4 Reverse Oligo: GACTACNVGGGTATCTAATCC] of 16S rDNA gene of bacteria and Archaea were used. The amplification of amplicons with the Illumina adaptors were performed by i5 and i7 primers that add multiplexing index sequences as well as common adapters required for cluster generation (P5 and P7) as per the standard Illumina protocol. The purification of amplicon libraries was done by 1 × AMpureXP beads and checked on Agilent DNA 1000 chip on Bioanalyzer 2100 and quantified on fluorometer by Qubit dsDNA HS Assay kit (Life Technologies). The library size of Sample LS and Sample SS were 2 million reads each. The sequencing of the libraries was done using the Illumina sequencing chemistry to generate ~150 Mb of data per sample. After obtaining the Qubit concentration for the library and the mean peak size from Bioanalyzer profile, library was loaded onto Illumina Platform at appropriate concentration (10–20 pM) for cluster generation and sequencing. The PCR amplicons were tagged with complementary adapter oligos on paired-end flow cell using the kit reagents. The designing of adapters was done as such to allow selective cleavage of the forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand was then used to perform sequencing from the opposite end of the fragments.

2.1. Bioinformatics analysis

The quality control of raw reads was performed by using FASTQC toolkit (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) [3]. The processed paired end reads were clustered into OTU's (Operational Taxonomic Units) by using QIIME software (qiime.org) in order to identify the microbial community profile [4]. The OTU's were further processed for taxonomic assignment (Greengenes database), phylogenetic and diversity analysis [5]. A large plethora of microbial communities were identified from this study.

Metagenome sequence data from this study are available at the NCBI Sequence Read Archive (SRA) and BioSamples under accession numbers: SAMN11571463 and SAMN11571474.

Funding

The work is supported by the funds provided to Dr. Mahashweta Mitra Ghosh and Dr. Sayak Ganguli by West Bengal DST project 236 (Sanc./ST/P&T/1G-39/2017).

Acknowledgement

The authors hereby acknowledge the Department of Microbiology, St. Xavier's College, Kolkata for providing the necessary facilities for conducting this study.

Conflict of interes

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] K. Ghosh, S. Mandal, Antibiotic resistant bacteria in consumable fishes from Digha coast, West Bengal, India, Proc. Zool. Soc. 63 (1) (2010) 13–20. https://doi.org/10.1007/s12595-010-0002-8.
[2] N. Garcias-Bonet, J.M. Arrieta, C.N. de Santana, C.M. Duarte, N. Marbà, Endophytic bacterial community of a Mediterranean marine angiosperm (*Posidonia oceanica*), Front. Microbiol. 3 (2012) 342. https://doi.org/10.3389/fmicb.2012.00342.
[3] S. Andrews, FastQC: a quality control tool for high throughput sequence data, Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc, 2010.
[4] J.G. Caporaso, J. Kuczynski, J. Stombaugh, K. Bittinger, F.D. Bushman, E.K. Costello, N. Fierer, A.G. Pēa, J.K. Goodrich, J.I. Gordon, G.A. Huttley, S.T. Kelley, D. Knights, J.E. Koenig, R.E. Ley, C.A. Lozupone, D. McDonald, B.D. Muegge, M. Pirrung, J. Reeder, J.R. Sevinsky, P.J. Turnbaugh, W.A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, R. Knight, QIIME allows analysis of high-throughput community sequencing data, Nat. Methods 7 (5) (2010) 35–336. https://doi.org/10.1038/nmeth.f.303.
[5] D. McDonald, M.N. Price, J. Goodrich, E.P. Nawrocki, T.Z. DeSantis, A. Probst, et al., An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea, ISME J. 6 (2012) 610–618. https://doi.org/10.1038/ismej.2011.139.

J. Environ. & Sociobiol. : **17**(1) : 1-10, 2020 Print : ISSN : 0973-0834
Received : 03 March, 2020 / Reviewed & Accepted : 10 June, 2020 / Uploaded Online : August, 2020

ANTIMICROBIAL AND COMPUTATIONAL ASSESSMENT OF *CHRISTELLA DENTATA* CRUDE EXTRACTS AGAINST MULTIDRUG RESISTANT BACTERIAL CULTURES AND TARGETS

Meesha Singh[1], Sayak Ganguli[2] and Mahashweta Mitra Ghosh[1*]
[1]Department of Microbiology, St. Xavier's College, Kolkata-700016
[2]Department of Biotechnology, St. Xavier's College, Kolkata-700016
*Corresponding author: mmgxaviers@gmail.com

ABSTRACT

There has been an abrupt increase in the emergence of antibiotic resistant bacteria and related infections throughout the globe. There is a dire need to explore novel botanicals with the purpose of identifying potential antibacterial compounds effective against such drug resistant bacteria. This study focuses on the screening of crude ethanolic extracts of an edible fern, *Christella dentata*. The biochemical tests and antibacterial efficacy assay against cultures of *E. coli*, *Salmonella*, *Pseudomonas*, *Bacillus* and *Lactobacillus* indicate the potency of the crude extracts. The study was further extended by text and literature-based identification of active principles from the plant under study. The identified compounds were then tested for their druggabilities and docked against reported targets of multidrug resistant (MDR) strains of *E. coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. Results indicate that some of these active compounds can be explored as potential leads for inhibiting the protein targets and preventing the spread of these MDR strains.

Key words: *Ferns, Hospital effluent, Multidrug Resistance, Phytochemicals, Molecular docking, Computational Study.*

INTRODUCTION

The antibiotic resistance in clinical pathogens is reportedly increasing at an alarming rate. This, in turn, is jeopardizing the sustainability of antibiotic use in human and veterinary medicine. For example, resistance to penicillin, ranged from zero to 51%, while that to ciprofloxacin caused by *E. coli* ranged from 8% to 65%

(WHO Report, 2014). The emergence of microbiological contaminants is still less investigated in developing countries. Drug resistance poses threat to global health that involves all major microbial pathogens and antimicrobial drugs (Olayinka *et al.*, 2009). Excessive use/misuse of antibiotics has led to the acquisition of resistance among microorganisms (Usha *et al.*, 2010). The re-emergence of old infectious diseases is also contributed by this phenomenon. The rate of prevalence of drug-resistance among bacteria is not proportional to the rate of development of new antimicrobial agents (Prescott and Klein, 2002). Changes in the genetic constitution of these resistant bacteria are said to be so rapid that the effectiveness of antibiotics may be lost within a period of 5-10 years (WHO report, 2014).

Therefore, it is crucial to look for alternative sources which present less/no resistance (Sarkar *et al.*, 2003). In order to develop alternative antimicrobial drugs, screening of plants is now being conducted throughout the globe (Chandra *et al.*, 2017). There are several reports that show satisfactory results with plants as antimicrobials (Vaghasiya *et al*., 2008; Fisgin *et al*., 2009; Jeong *et al*., 2009; Darwish and Aburjai, 2010; Yoon *et al*., 2011).

Phytochemicals with recognized antibacterial activity belong mainly to the following chemical structural classes: phenolics, terpenoids and other essential oil constituents, alkaloids, lectins and polypeptides etc. The major subclasses are: simple phenols and phenolic acids, quinones, flavones, flavonoids and flavonols, tannins, coumarins, terpenoids and essential oils, alkaloids, lectins, isothiocyanates, stilbenes etc. (Cowan, 1999; Dorman and Deans, 2000; Gibbons, 2004; Newman *et al*., 2000; Stavri *et al*., 2007).

Bioactivities of plant antimicrobials against following mechanisms: inhibition of biofilm formation, efflux-pump inhibition, attenuation of bacterial virulence etc., responsible for resistance to multiple drugs, are in report (Dalleau *et al*., 2008; Khan and Ahmed, 2012; Nostro *et al*., 2007).

Screening of crude plant extracts for synergistic interaction with antibiotics can help us to identify bioactive compounds to be used in combinational therapy. Various Bioinformatics tools, programs and servers can aid in identification of different active compounds from plants having antimicrobial potential and interactive visualization and analysis of molecular structures. Subsequent identification and purification of novel anti-microbial phytocompounds followed by molecular docking against proteins of potential multi-drug resistant bacteria can help to design new drugs combining both antibiotics and phytocompounds to treat infectious diseases.

MATERIAL AND METHODS

Sample collection: Fronds and rhizomes of *Christella* species were collected from the Lepchakka village (North Bengal) area located at around 3500 ft. The samples collected were stored in sterile bags and stored at -20°C after transportation.

Extraction: The leaves and roots were dried separately. The dried materials were grinded to fine powder by a domestic grinder and stored in moisture free containers till further use. The extracts of different plant parts were prepared using ethanol. The concentration of the final extracts was 10 mg/ml.

Biochemical Assay

DPPH Radical Scavenging Activity: 1 ml of DPPH solution was added to 1ml of the dilute extract of each sample. The mixture was shaken vigorously and left to stand at room temperature for 20 mins in dark. The absorbance was measured at 517 nm against a blank in UV/Vis spectrophotometer. The percentage of DPPH discoloration of the sample was calculated according to the equation: % discoloration = (1 –Abs sample / Abs control) *100.

Total Polyphenol Content: 500µl of 1:10 FC reagent was mixed with 1 ml of the dilute extract. 4 ml of 7% Na_2CO_3 solution was added to it. Then the mixture was allowed to stand for 30 mins. The resulting blue complex was then measured at 765 nm against a blank.

Total Flavonoid Content: 1ml of extract was mixed with 0.3 ml of 5% $NaNO_2$ and 0.3 ml of 10% $AlCl_3$. Then this mixture was kept at room temperature for 5 min. 2 ml of NaOH and 1ml of H_2O were added to the mixture. The mixture was then allowed to stand for 30 min and then the absorbance was taken at 510 nm against a blank.

Tannin Content: 200 µl of the extract was mixed with 1.5 ml of 4% vanillin. Then 0.75 ml of conc. HCl was added to the mixture and allowed to react at room temperature for 20 mins. The absorbance was then taken at 500 nm against a blank.

Antimicrobial Assay: Nutrient agar plates were spread with different strains of *E. coli*, *Salmonella*, *Pseudomonas*, *Bacillus* and *Lactobacillus.* The anti-bacterial activities of the plant extracts were tested by bore-well method with 200 µl ethanol as control. The plates were then kept in the B.O.D incubator overnight.

Computational Analyses

The following targets were studied:

- **5TKW**: 1.35 Angstrom Resolution Crystal Structure of a Pullulanase-specific Type II Secretion System Integral Cytoplasmic Membrane Protein GspL (N-terminal fragment; residues 1-237) from *Klebsiella pneumoniae*.
- **2XCQ**: The 2.98A crystal structure of the catalytic core (B'A' region) of *Staphylococcus aureus* DNA Gyrase.
- **4E81:** Crystal structure of the substrate binding domain of *E. coli* DnaK in complex with a short apidaecin peptide.

Crystal Structures of 5TKW, 4E8I and 2XCQ were downloaded from the Protein

Data Bank [https://www.rcsb.org/]. Details of the small molecules of *Christella* were studied from literature and noted. These were then searched for in PUBCHEM database [https:/ pubchem.ncbi.nlm.nih.gov/] and the list of compounds were curated. The compounds were downloaded in .sdf format and converted to .pdb using the online SMILES translator tool [https:// cactus.nci.nih.gov/translate/]; following this they were tested for their druggability and bioactivity using the MOLINSPIRATION server [http://www.molinspiration.com/] (Ganguli *et al.*, 2011; Salma *et al.*, 2017). The PatchDock server [https://bioinfo3d.cs.tau.ac.il/PatchDock/] was used to analyze the propensity of complex formation through molecular docking and the atomic contact energies were noted (Duhovny *et al.*, 2005). Protein-ligand complexes were then analyzed for their interactions using Ligplot. [https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/] (Wallace *et al.*, 1995).

List of active compounds from *Christella*:

- Beta sitosterol
- Hexacosyl hexadecanoate
- Kaempferol
- Matteucinol

RESULTS AND DISCUSSION

Biochemical Assays: Ethanolic extracts of *Christella* roots displayed higher free radical scavenging activity as compared to the leaves (Fig. 1). This has been previously reported by Rekha, 2017. Antioxidant property of eight fern species from North of Iran have also been reported and found that rhizome extract were stronger radical scavengers than the aerial part extract in all the ferns which is in conformation with the results of current investigation.

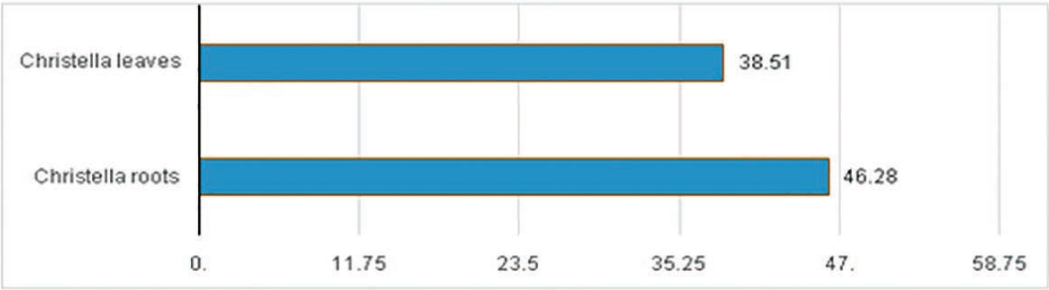


Fig. 1. DPPH radical scavenging potential (%)

Polyphenol and Flavonoid contents were found to be higher in case of *Christella* leaves as compared to the rhizome and roots (Fig. 2); however, total tannin content was found to be higher in the *Christella* rhizome and roots. Most of the previous studies have denoted that the presence of secondary metabolites in rhizome and roots is higher than that in leaves of ferns (Britto *et al*., 2012; Santos *et al.*, 2010).

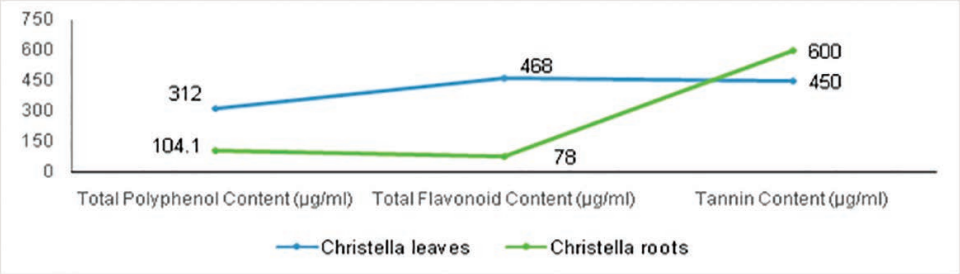


Fig. 2. Biochemical assay results

Antimicrobial Assay: The zone of inhibition was measured to identify the potency of the extracts. Antimicrobial assay results indicate that root extracts have higher efficacy towards inhibiting the microorganisms under study. It was found to be effective against *E. coli*; *Bacillus*, *Lactobacillus* and *Salmonella* but not against *Pseudomonas*. On the other hand, Leaf extracts were found to inhibit *Bacillus*, *Pseudomonas* and *Salmonella* effectively.

Table 1. Antimicrobial assay (Zone of inhibition data)

Bacterial culture	Zone of Inhibition (cm)	
	C. leaves	C. rhizome
E. coli	0	1
Bacillus	0.9	1.1
Lactobacillus	0	1
Pseudomonas	0.8	0
Salmonella	1	1.2

Computational Analyses: Results from the screening of the compounds for their drug likeliness and bioavailability revealed that most of the compounds analyzed were suitable as potential leads since they did not violate the Lipinski's Rule of five (Lipinski *et al.*, 2001) by more than one parameter. However, Hexacosyl hexadecanoate was found to have a higher molecular weight than the acceptable limit and would need suitable chemical modifications if selected. The compounds were then docked with the selected targets and their atomic contact energies were noted. For 5TKW the best combinations of energy and area of interaction was found to be with Beta sitosterol; for 2XCQ it was Kaempferol and for 4E81; Hexacosyl hexdecanoate proved to be the best interacting molecule. (Figs. 3, 4, 5).

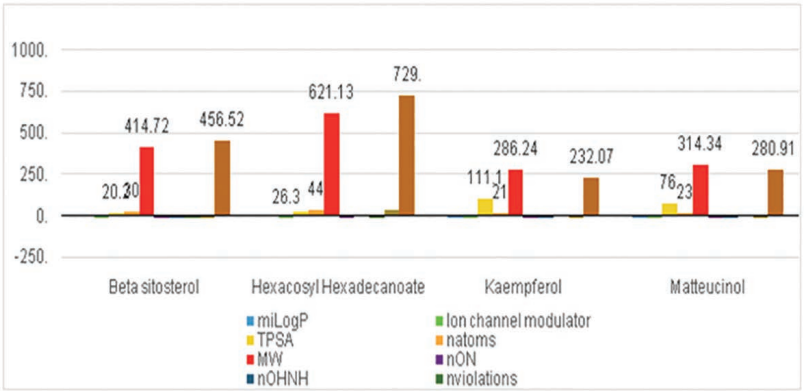


Fig. 3. Molinspiration data

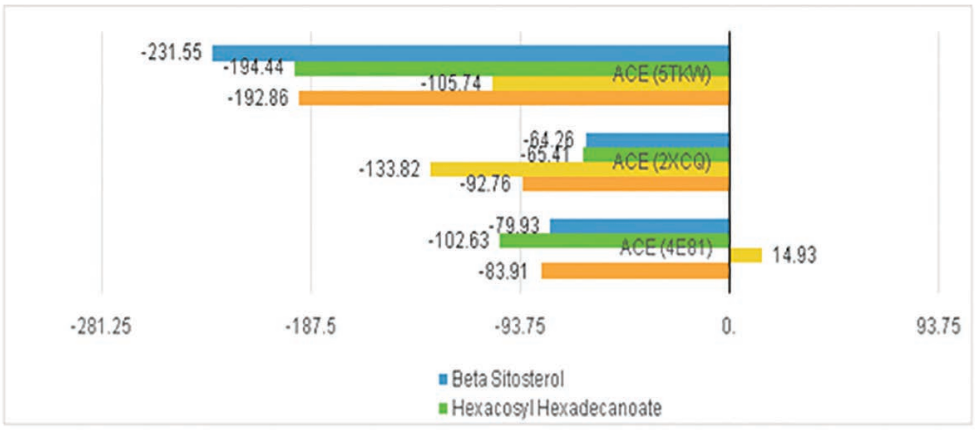


Fig 4. Atomic Contact Energy (ACE) values

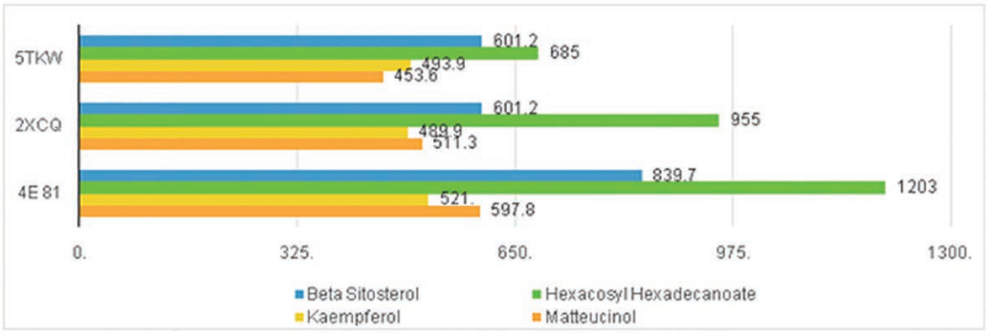


Fig. 5. Area of docking

Molecular docking results: The analyses of the interaction complexes of the best results revealed that hydrogen bonded and non-bonded interactions were together regulating the complex formation. In case of 5TKW- Beta sitosterol interaction 2 hydrogen bond(s) having distance of 2.77 angstrom and 3.02 angstrom along with 49 Van der Waals interactions were identified whereas 2XCQ-Kaempferol interactions revealed 2 hydrogen bonds with a distance of 2.82 angstrom and 2.91 angstrom and 12 Van der Waals contacts (Figs. 6 and 7).

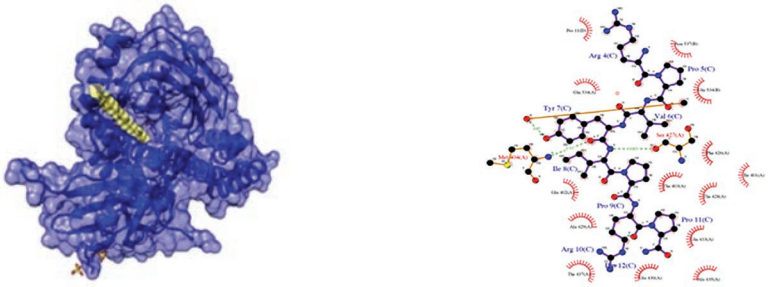


Fig. 6. 5TKW- Beta sitosterol: (a) Docked compound; (b) Hydrogen bond and Van der Waals interactions

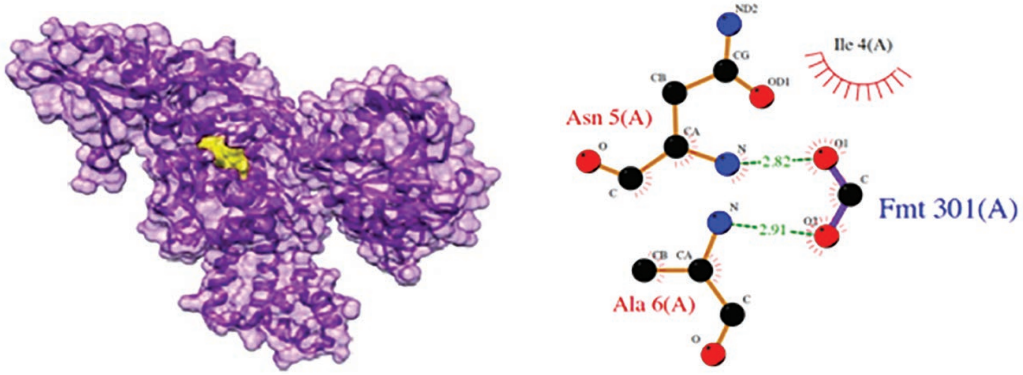


Fig. 7. 2XCQ- Kaempferol (a) Docked compound; (b) Hydrogen bond and Van der Waals interactions

The present investigation based on the biochemical and antimicrobial analysis of *Christella* leaves and roots shows strong efficacy of their extracts in biochemical as well as in antimicrobial assays (Kumar and Kaushik, 2011; Ghosh *et al.*, 2004). The average free energies of the interactions were found to be -231.55 Kcal/mole for 5TKW-Beta sitosterol complex, -133.82 Kcal/mole for 2XCQ- Kaempferol and -102.63 Kcal/mole for 4E81-Hexacosyl hexadecanoate complex which are indicative of stable complex formation.

CONCLUSION

Thus, from the above data we can conclude that some of the compounds under study displayed affinity towards binding to the target proteins with stable complex formation as indicated by the interaction energies and thus can be further tested experimentally for their establishment as leads for drug discovery. Subsequent identification and purification of novel anti-microbial phytocompounds followed by docking against proteins of potential multi-drug resistant bacteria can help to design new drugs combining both antibiotics and phytocompounds to treat infectious diseases.

ACKNOWLEDGEMENTS

The authors hereby acknowledge WB-DST [Memo no.: 236 (Sanc.) /ST/P/S&T/1G-39/2017] for the financial support. The authors also acknowledge Department of Microbiology, St. Xavier's College (Autonomous), Kolkata for the provision of necessary facilities. Meesha Singh (Junior Research Fellow) is thankful to the WBDST for her research fellowship.

AUTHORS CONTRIBUTION

MS performed the experiments. SG designed the study and MMG supervised the work and finalised the manuscript

ETHICS APPROVAL, CONSENT TO PARTICIPATE AND PUBLISH

All the authors hereby declare that the manuscript is original, authentic and is ethically upright and they have full consent towards the publication of this article.

CONFLICT OF INTEREST

Authors hereby declare that there is no conflict of interest associated with this work

REFERENCES

Britto, J. D., Gracelin, H. S. and Kumar, P. B. 2012. Phytochemical studies on five medicinal ferns collected from Southern Western Ghats, Tamil Nadu. *Asian Pac. J. Trop. Biomed*., **2**: 536-538.

Chandra, H., Bishnoi, P., Yadav, A., Patni, B., Mishra, A. P. and Nautiyal, A. R. 2017. Antimicrobial resistance and alternative resources with special emphasis on plant-based antimicrobials - a review. *Plants (Basel),* **6**(2): 16.

Cowan, M. M. 1999. Plant products as antimicrobial agents. *Clin. Microbiol. Rev*., **12**(4): 564-582.

Dalleau, S., Cateau, E., Berges, T., Berjeaud, J. M. and Imbert, C. 2008. *In vitro* activity of terpenes against Candida biofilms. *Int. J. Antimicrob. Agents*, **31**(6): 572-576.

Darwish, R. M. and Aburjai, T. A. 2010. Effect of ethnomedicinal plants used in folklore medicine in Jordan as antibiotic resistant inhibitors on *Escherichia coli*. *BMC Complement. Altern. Med.,* **10**: 9-16.

Dorman, H. J. D. and Deans, S. G. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J. Appl. Microbiol*., **88**(2): 308-316,

Duhovny, D., Inbar, Y., Nussinov, R. and Wolfson, H. 2005. PatchDock and SymmDock: servers for rigid and symmetric docking. *Nucleic Acids Res.*, **33**: 363-367.

Fisgin, N. T., Cayci, Y. T., Coban, A. Y., Ozatli, D., Tanyel, E., Durupinar, B. and Tulek, N. 2009. Antimicrobial activity of plant extract Ankaferd Blood Stopper®. *Fitoterapia,* **80**(1): 48-50.

Ganguli, S., Datta, A. and Gangopadhyay, A. 2011. Inhibiting h5n1 haemagglutinin with small molecule ligands. *Int. J. of Bioi. Res.*, **3**(1): 185-189.

Ghosh, S. R., Ghosh, B., Biswas, A. and Ghosh, R. K. 2004. The Pteridophytic Flora of Eastern India. *Flora of India Series 4. Botanical Survey of India*, **1**: 54-59.

Gibbons, S. 2004. Anti-staphylococcal plant natural products. *Nat. Prod. Res*., **21**(2): 263-277.

Jeong, M-R., Kim, H-Y. and Cha, J-D. 2009. Antimicrobial activity of methanol extract from *Ficus carica* leaves against oral bacteria. *J. Bacteriol. Virol.,* **39**(2): 97-102.

Khan, M. S. A. and Ahmad, I. 2012. Biofilm inhibition by *Cymbopogon citratus* and *Syzygium aromaticum* essential oils in the strains of *Candida albicans*. *J. Ethnopharmacol*., **140**(2): 416-423.

Kumar, A. and Kaushik, P. 2011. Antibacterial activity of *Christella dentata* Forsk. Study in different seasons. *J. Chem. Pharm. Res*., **3**(6): 153-158.

Levy, S. B. 2002. *The Antibiotic Paradox: How the Misuse of Antibiotics Destroys Their Curative Powers.* Cambridge, MA: Perseus Publishing.

Lipinski, C. A., Lombardo, F., Dominy, B. W. and Feeney, P. J. 2001. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev*., **46**(1-3): 3-26.

Newman, D. J., Cragg, G. M. and Snader, K. M., 2000. The influence of natural products upon drug discovery. *Nat. Prod. Rep.*, **17**(3): 215-234.

Nostro, A., Roccaro, A.S., Bisignano, G., Marino, A., Cannatelli, M. A., Pizzimenti, F. C., Cioni, P. L., Procopio, F. and Blanco, A. R., 2007. Effects of oregano, carvacrol and thymol on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. *J. Med. Microbiol*., **56**(4): 519-523.

Olayinka, A. A., Anthony, J. A. and Anthony, O. I. 2009. Synergistic interaction of *Helichrysum pedunculatum* leaf extracts with antibiotics against wound infection associated bacteria. *Biol. Res.,* **42**(3): 327-338.

Prescott, H. and Klein, J. O. 2002. *Microbiology,* 6th ed. Macgraw Hill Publishers, USA*,* pp., 808-823.

Rekha, K. 2017. Preliminary Phytochemical Analysis and Antioxidant Property of the fern, *Christella dentata* (Forssk.) Brownsey & Jermy. *WJPLS*., **3**(1): 146-150.

Salma, A., Sherbeni, EI, Tarek, F and Moselhy, EI. 2017. Computational evaluation of the druggability and biological activity of Iodo-1,4-dihydropyridine derivatives. *Chemistry Research Journal,* **2**(5): 131-141

Santos, M. G., Kelecom, A., Paiva, S. R., Moraes, M. G., Rocha, L. and Garrett, R. 2010. Phytochemical studies in pteridophytes growing in Brazil: a review. *Americas J. Plant. Sci. Biotech.,* **4**: 113-125.

Sarkar, A., Kumar, K. A., Dutta, N. K., Chakraborty, P. and Dastidar, S. G. 2003. Evaluation of *in vitro* and *in vivo* antibacterial activity of dobutamine hydrochloride. *Indian J. Med. Microbiol.,* **21**(3): 172-178.

Sharma, L. and Kumar, A. 2006. Antimicrobial activity of *Ageratum conyzoides* Linn. a plant with extra-medicinal value. *Asian J. Exp. Sci.*, **20**: 41-46.

Stavri, M., Piddock, L. J. V. and Gibbons, S. 2007. Bacterial efflux pump inhibitors from natural sources. *J Antimicrob Chemother*., **59**(6): 1247-1260.

Usha, P. T. A, Jose, S. and Nisha, A. R. 2010. Antimicrobial drug resistance –A global concern. *Vet. World,* **3**(3): 138-139.

Vaghasiya, Y., Nair, R. and Chanda, S. 2008. Antibacterial and preliminary phytochemical and physico-chemical analysis of *Eucalyptus citriodora* Hk leaf. *Nat. Prod. Res.,* **22**(9): 754-762.

Wallace, A. C., Laskowski, R. A. and Thornton, J. M. 1995. LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. *Protein Eng*., **8**(2): 127-134.

World Health Organization. 2014. Antimicrobial resistance: global report on surveillance, **1**: 35-40.

Yoon, J.I., Bajpai, V.K., Kang, S.C. 2011. Synergistic effect of nisin and cone essential oil of *Metasequoia glyptostroboides* Miki ex Hu against *Listeria monocytogenes* in milk samples. *Food Chem. Toxicol.,* **49**(1): 109-114.

A Pipeline for Assessment of Pathogenic Load in the Environment Using Microbiome Analysis

23

Subhoshmita Mondal, Sohini Gupta, Meesha Singh, Somosree Pal, Kaustav Das, Mahashweta Mitra Ghosh, Subrata Sankar Bagchi, and Sayak Ganguli

Abstract

The contamination of the environment is taking place at a very fast pace. Industrial pollutants, hospital effluents, domestic and household wastes as well as direct environmental processes such as melting of ice caps around the globe with increase in mean temperature as a result of global warming are contributing towards the continuous alterations in environmental balance. The threats of natural calamities such as cyclones and earthquakes have also challenged the habitability of many areas. As a result pandemics such as COVID-19, Ebola, and at a lesser scale dengue and malaria are affecting a large global population. It is thus important for epidemiologist and public health sectors to come up with suitable prediction models of the pathogenic load of a particular area so as to design and implement suitable mitigation measures. In this work we propose a metagenomics assisted pipeline for estimation of pathogenic load in the

S. Mondal
Department of Chemical Engineering, Jadavpur University, Kolkata, India

S. Gupta
Department of Botany, Barasat Government College, Barasat, India

M. Singh · M. M. Ghosh
Department of Microbiology, St. Xavier's College (Autonomous), Kolkata, India

S. Pal · S. S. Bagchi
Department of Anthropology, University of Calcutta, Kolkata, India

K. Das
Department of Anthropology, Bangabasi College, Kolkata, India

S. Ganguli (✉)
Department of Biotechnology, St. Xavier's College (Autonomous), Kolkata, India

M. Nath et al. (eds.), *Microbial Metatranscriptomics Belowground*,
https://doi.org/10.1007/978-981-15-9758-9_23

environment with case studies from rhizospheric soil microbiome, effluent and wastewater microbiome, and human gut microbiome, where we perform a microbiome wide association study with known disease causing microbial datasets and predict the potential pathogenic microorganisms that are prevalent in a particular area or ecological niche. Our pipeline was able to predict the potential pathogenic load of the niche areas under study, which leads us to believe that metagenomics can be utilized at a diagnostic scale and using the dataset obtained we may then predict the pathogenic load of that particular area. This approach has the potential to be utilized for fast prediction of potential disease threats under public health emergencies and should enable proper resource partitioning from suitable stakeholders.

Keywords

Metagenomics · Rhizosphere · Wastewater · Effluent · Gut Microbiome

23.1 Introduction

We are currently in the era of microbes. Permafrost is melting, thus releasing high volumes of unknown microbes into the environment which were trapped into the sub-zero temperatures. We are currently facing an international pandemic with the severity which the world witnesses every 100 years and yet we are doing very little to establish and fast pace diagnostics and monitoring pipeline for pathogenic load that is being released in to the environment. A few repositories have been established by synchronizing the diversity which include the Human Microbiome Project (Micah et al. 2007), the Tara Ocean Project (Karsenti et al. 2011), and the Earth Microbiome Project (Gilbert et al. 2014). Metagenomics have also shown its merit in helping to predict the ancient past from an organisms fossilized DNA (like bones, teeth). While many studies related to ancient DNA focus on the investigations of human endogenous DNA isolated from specimens of ancient ages (Haak et al. 2015, Mallick et al. 2016, Orlando et al. 2013, Schlebusch et al. 2012, Skoglund et al. 2017), associated environmental reconstructions can also be made which throws light on the microbial abundance of the past and also increase our understanding towards the evolution of infectious diseases (Warinner et al. 2017; Key et al. 2017). We know next-generation sequencing is a traditional method to analyze metagenomes, either through amplicon or shotgun sequencing. There are specifically three benefits of using 16S ribosomal RNA amplicon sequencing when compared to shotgun sequencing methodology. Using 16S ribosomal RNA amplicon sequencing is profitable, secondly, data can be analyzed by pre-established bioinformatics channels and the referral databases which are relatively comprehensive (Ranjan et al. 2016; Sedlar et al. 2017). On the other hand, two drawbacks of 16S rRNA amplicon sequencing are, it spots richness of a lower species; and classifies at phylum level, fairly, genus level. Comparatively, shotgun sequencing detects every position of strands of lower species level, and, identifies bacterial species more significantly, by recognizing greater diversity organisms of other kingdoms (Ranjan et al. 2016).

Diagnostics is an emerging area where metagenomics is applied and emphasized. As defined by Pallen, diagnostic metagenomics (Pallen 2014) recognizes and characterizes pathogens using shotgun metagenomic data. In a way, diagnostic metagenomics possess generic potential to ideally and swiftly trace all microbial (includes bacterial, viral, and parasitic) pathogens as well as infections caused due to respective microbes through many samples of feces, urine, meat, blood, etc. The advantage of diagnostic metagenomics is that, it reduces the time consumed from sampling to result to less than 24 h, instead of cultivation of pathogens for several days. There is also an excellent alternative method, i.e., polymerase chain reaction (PCR) enriched with pathogen specificity, but, it requires splitted PCR setup for every targeted pathogen. The features like vulnerability, specificness, capability of quick identification (also quantification in few cases) of pathogens allow metagenomics to play a key role in diagnostics. However, there is still more to explore about complete potential of diagnostic metagenomics at its experimental stages. Considerably, data analysis is an important part of metagenomic studies which is difficult as well as time consuming. The metagenomic sequences which either interpret or assemble are categorized on the basis of taxonomy dependent and taxonomy independent. The first classification, taxonomy dependent is based upon a referral database with sequenced data or only marker genes (Mande et al. 2012; Sedlar et al. 2017; Lindgreen et al. 2016; Menzel et al. 2016). In 2014, Wood and co-authors described the approach of taxonomy dependent used in different classifiers (Wood and Salzberg 2014). In 2015, Ounit et al., used the classifier CLARK (CLAssifier based on Reduced K-mers) (Ounit et al. 2015), simultaneously, in 2017, the usage of metagenomic mapper (MGmapper) was published in 2017 (Petersen et al. 2017). The metagenomic phylogenetic analysis (MetaPhlAn) is an example of a reference-based tool which only emphasize on a defined set of strain-specific marker genes (Segata et al. 2012). Meanwhile, all these taxonomy-dependent classifiers likely provide fewer details concerning the sequences, and the search against the database becomes much faster (Segata et al. 2013). Naccache et al., reported a resolute pipeline of bioinformatics for diagnostic metagenomics, named as, Sequence-based Ultra-rapid Pathogen Identification (SURPI) which is a reference-based pipeline and uses the National Center for Biotechnology Information (NCBI) nucleotide database and the RefSeq non-redundant proteins database in its comprehensive mode (Naccache et al. 2014). The methods classified under taxonomy-dependent metagenomic sequences are frequently used in diagnostic metagenomics because the integrated databases can rapidly detect the causative pathogen which is useful for further treatment of a patient. Presently, the majority of the accessible databases are inadequate and/or distorted to incorporate additional number of human pathogens and model organisms (Segata et al. 2013). Now, these unclassified sequences, raises the risk of false positive results, where non-pathogen with sequences similar to pathogens are detected and finally categorized as pathogens because of incorrect references (Ranjan et al. 2016; Sedlar et al. 2017). Many defined databases and networks are in the process of development by the U.S. Food and Drug Administration (for example, GenomeTrakr) (Allard et al. 2016) and to add to it are European Commission funded collaborative management

platforms (such as, COMPARE project) (Aarestrup and Koopmans 2016) for spotting and analytical studies of re-emerging food borne outbreaks. The second classification of metagenomics sequence is taxonomy independent which is also known as binning, only depends on the sequence composition based data (Sedlar et al. 2017; Sangwan et al. 2016). The approach of taxonomy-independent classification in metagenomic studies was used as compiler, for example, CONCOCT (Clustering Contigs with Coverage and Composition) (Albertsen et al. 2013; Nielsen et al. 2014; Cleary et al. 2015; Alneberg et al. 2014). However, it was reviewed that these studies and programs cannot be applied for diagnostic purposes (Sangwan et al. 2016). The classification of metagenomic sequences are made more precise and correct pre-processing of the shotgun data for quality control include trimming, masking, and assembly. However, assembling reads into contigs makes the analysis better, but, from bioinformatics point of view, it is usually a difficult task (Ranjan et al. 2016; Sedlar et al. 2017; Sangwan et al. 2016). Generally, it is suggested to examine the exposure of the metagenomic data set because higher exposure is helpful to assemble and detect distinctly abundant genes in a more systematic way (Rodriguez-r and Konstantinidis 2014). It was reviewed that many tools are available for end-to-end metagenomic data analysis together with pre-processing (Segata et al. 2013). Yun and Yun, in 2014 reported the comparison of the two pre-processing methods trimming and masking. It was observed that the method of masking is more commendable than trimming, because masking was better in analyzing the rate of false positive data of single nucleotide polymorphism, and, bases of low quality present in the sequence are replaced with "N" which are not detected. On contrary, trimming process is frequently used due to efficient removal of low quality bases (repeated only at the ends of a read) results into a shorter read (Yun and Yun 2014). Andersen et al., in 2017a, b focused on the importance of pointing out the loop holes of the software used for analysis. In a case study, fecal samples of ten-fold dilutions showed data spiked with *Campylobacter jejuni* which were detected by two taxonomic classifiers Kraken and CLARK. It was observed that both classifiers identified false positive reads from negative samples with scarcely present *Campylobacter jejuni* of quantitative polymerase chain reaction (qPCR). It was developed that sorting of Kraken hits can eliminate false positives reads. While sorting of Kraken hits, firstly, the sequences are sorted by assigning each hit a score, then, hits for phage and plasmid DNA are removed using the Kraken and Basic Local Alignment Search Tool (BLAST) among the high scoring hits (Andersen et al. 2017a, b). The study also represented a non-linear correlation between the rising levels and the hits read from metagenomic data.

23.2 General Analysis Pipeline

The pipeline involves the use of advanced next generation sequencing techniques, though traditional methods are also in use, while Table 23.1 summarises the general approaches for pathogen detection from samples.

Table 23.1 Strategy and Detection of Pathogens from different samples (modified from Miller et al. 2013)

Application/ Strategy	Method	Application	Examples	Advantages	Disadvantages
Deep sequencing	rRNA	Identification of prokaryotes and eukaryotes	Human gut microbiome characterization	Highly sensitive	Concerns regarding universality of target gene
		Determination of taxonomic relationships	Similarities of ancient gut with modern rural gut and dissimilar with modern urban gut	Taxonomic classification can be done using fewer reads	Primer bias may be detrimental
	rpoB	Archaeal and bacterial identification*	Applicable to subgroup species	rpoB and cpn-60 offer enhanced taxonomic resolution compared to rRNA	Possibility of variable gene copy numbers among targeted species
	Cpn-60	Taxonomic relationships determination			
	Viral RNA polymerase (RdRP)	Novel virus discovery	Identified novel families of picornaviruses off the coast of British Columbia		
Metagenomics	Shotgun sequencing	Functional and taxonomic characterization	Swine fever virus like sequences were detected(Asfarviridae)	Microorganism wide detection of sequences	Broad specificity may lead to decreased sensitivity
			Stool samples exhibited the presence of unexpected microbes	No a priori knowledge of microorganisms required	Labor intensive detection of sequences
	Subtraction	Functional and taxonomic characterization	Identified divergent regions in non-coding RNAs in Listeria monocytogenes	Potential for bias is reduced by the use of random primers	Bioinformatics analysis is more challenging
			Association of *Fusobacterium nucleatum* with colorectal carcinoma		Relatively expensive as more reads are required

(continued)

Table 23.1 (continued)

Application/ Strategy	Method	Application	Examples	Advantages	Disadvantages
	Virus	Novel virus discovery	Detection of the novel H1N1 influenza from nasopharyngeal swabs		Almost 50% of the generated sequences generally have no significant homology to known proteins in databases (dark matter)

Once the abundance data is obtained then the comparative metagenomics predictions are initiated using tools such as Venny and Comparator. These provide us with a set of unique and common dataset for each of the comparative datasets that are being evaluated. Following the identification of the subset the metagenome-wide association studies are initiated which is mainly focused on taxonomic enrichment analysis. Metagenome-wide association studies have already found strong association of microbes with host health and disease. It also identifies a large number of microbes differentially regulated in various conditions. However, computational methods for analyzing such differentially regulated microbes from microbiome study are limited. TSEA or Taxon Set Enrichment Analysis is a way to identify biologically or ecologically meaningful patterns by analyzing them with context to pre-defined taxon set (microbes sharing some common trait) from a given list of significant features or microbes. These microbes undergo certain significance levels and the obtained results are combined to observe discerned meaningful patterns. In contrast, TSEA directly examines a set of functionally related microbes without any preselected compounds based on arbitrary cutoff threshold. TSEA has potential to identify subtle but consistent changes among a group of related microbes, which may go undetected with conventional approaches.

23.3 TSEA Overview

Taxon set enrichment analysis comprises 4 steps of data assembly—input, processing, analysis, and compilation of results. Microbiome Analyst Different taxon sets are selected on the basis of different input types and supported by three types of taxon sets which are based on the taxonomic resolution of microbes to be analyzed. The taxon name mapping to higher taxonomic level of variety of microbes by using major database identifiers can be performed by users. TSEA offers three algorithms for enrichment analysis with three different data inputs required for following three approaches:

1. A list of microbes are characterized at any possible taxonomic level—entered as a one column data (Mixed-level taxa);
2. A list of microbes are characterized at any species level taxa and enlisted in one column (Species level taxa).
3. A list of microbes names (Binomial Nomenclature Name/GOLD ID/NCBI Taxonomy ID) characterized at any strain level—entered as a one column data (Strain-level taxa)).

23.4 Selection of Taxon Set Library

Our entire list was analysed using a mixed level taxon set. Mixed-level taxon sets associated with Human diseases were used for improved analysis. Over Representation Analysis (ORA) is done by enlisting taxa or microbes found to be abundant in

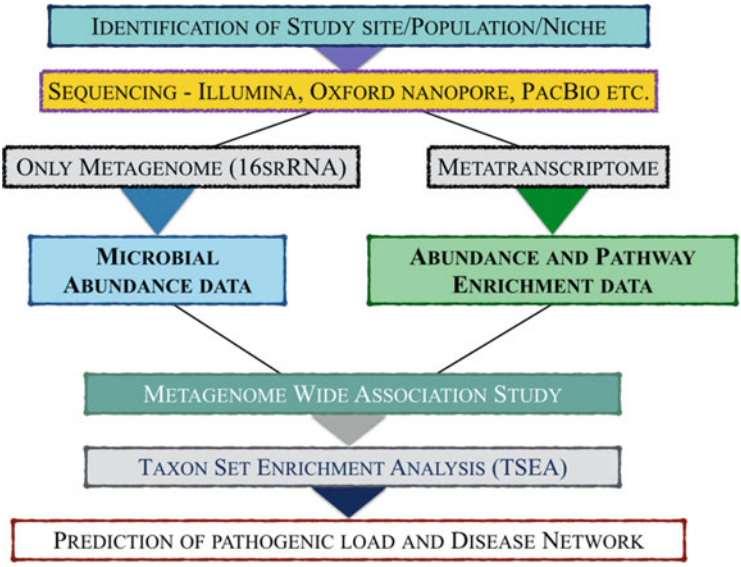


Fig. 23.1 Proposed pipeline for detection of pathogenic load in a an environmental niche

the individual data sets and common to all. The list of microbes can also be obtained through differential abundance testing, or from biomarker analysis or from clustering of algorithm to examine a few biologically meaningful patterns, if present. ORA was implemented using hyper-geometric test to calculate whether a particular taxon set is represented more than expected by chance within the given list. One-tailed p values are generated after adjusting multiple tests. Figure 23.1 summarizes the proposed pipeline for identification of pathogenic load.

23.5 Case Reports

There are numerous microbiome analysis reported on the applications of metagenomics. The following section will focus on a few existing data.

23.5.1 Case Study 1

One of the case studies reported in recent past showed the use of metagenomics in diagnosis of clinical fecal samples. The sampling was done from individuals of two categories, the patients with illness and the patients recovered after 3 months. The data was analyzed by Nucleotide Basic Local Alignment search tool (BLAST) against a reference database. *C. jejuni* was identified as the pathogen from the samples collected from patients suffering from illness because the reads of the analysis aligned to *C. jejuni*. However, the pathogen was detected through

metagenomic data and diagnosed, the study was very limited to the dependency of the samples collected from the patients after 3 months recovery and the method was inapplicable to real-time surveillance situations (Nakamura et al. 2008). Loman et al., performed an experiment with 45 human fecal samples while a breakout in Germany in 2011of Shiga-toxigenic Escherichia coli *O104:H4. Among 45 samples considered for the study, 40 were observed to have pathogen. 45 samples were paired end to end and sequenced with 151 bp to yield total 180 giga base pairs using HiSeq (Illumina). It was likely to retrieve a draft of genome of strains obtained from 27 human fecal samples collected during the outbreak. In addition, genes of* Escherichia coli *O104:H4 were identified from 27 human fecal samples (Loman et al. 2013).*

23.5.2 Case Study 2

In 2016, Schneeberger et al., using shotgun sequencing demonstrated a proof of application of diagnostic metagenomics. For the experiment, 4 fecal samples were collected from patients suffering with persistent diarrhea. The patients were from areas of high occurrence of gastrointestinal infections with asymptomatic carriers and co-infections. The samples were observed to have bacterial, parasitic, and viral infections. The comparison analysis of data was carried out by BLASTn against three reference reads from NCBI databases: nucleotide, genome-specific markers (GSMer), and inclusive antibiotic resistance database (CARD). Each patient with 8–11 different pathogens was detected to be positive, which was more than if diagnosed with conventional methods like, microscopy, cultivation, and multiplex PCR (Schneeberger et al. 2016). The result imposed question on how many infectious pathogens and asymptomatic carriers were detected, and, how many pathogens were detected with false positive hits. Nevertheless, the study showed the potential of taxonomy-dependent method which used the entire genome sequence, markers and Antibiotic Resistance Genes(ARGs) to detect pathogens and co- infections from multiple classes of kingdoms.

23.5.3 Case Study 3

The plant microbiome group has been extensively studied the microbial composition of rhizospheric soils of several plants from the Indian Sunderbans. Indian Sunderbans represent the deltaic region of the rivers Ganga and Brahmaputra (India) and Meghna (Bangladesh). These are uniquely characterized as they are under continuous tidal inundation and agricultural practices are very limited to local landraces of rice and a few leafy vegetables due to the high salt content of the soil. The region is also plagued with geographical challenges and unorganized healthcare facilities. Due to the prevalent high humidity conditions, flu, dengue, as well as other diarrheal diseases are very common. The rhizopsheric microbial abundance was used as the starting data and pathogenic load around human habitats

were predicted as described in the material and method segment. We found that several pathogenic microbes were identified having reported pathogenesis in Colitis and malaria (Ganguli et al. 2017, Rahaman et al. 2019). Apart from that several antibiotic resistance pathways were also found to be upregulated such as beta lactam resistance, vancomycin resistance, and neomycin resistance (Fig. 23.2).

Disease network analysis (Fig. 23.3) revealed the interactions of the causative pathogens and the diseases that share the pathogen as causal agents. However, as it can be observed that complex disease networks are not very prevalent in the analysis which indicates that the pathogenic load of the area under study is moderate.

23.5.4 Case Study 4

The evolution of new strains with antibiotic resistivity is gradually affecting public health with implications on economic and social throughout the world. The infections like pneumonia, typhoid fever, etc., are caused by *Streptococcus*, which are community acquired infections. The infections caused due to methicillin (antibiotic) resistance of *Staphylococcus aureus*, vancomycin resistance of *Enterococci,* and various other Gram-negative bacteria producing beta-lactamase enzyme producing Gram-negative bacteria, are known to be hospital acquired infections. These infections direct additional diseases to patients with their longer stay at hospitals which may cause pressure ulcers (bedsore) and economic burden on the community. The common organisms identified during pressure ulcers are *Staphylococcus aureus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis*. The emergence of pathogenic bacteria showing resistivity towards most of the currently available antimicrobial agents has really become a critical problem in area of modern medicine, particularly because of the increase in immune suppressed patients. In June 2000, WHO (World Health Organization) warned regarding the increase in the level of resistivity of drugs towards treating common infectious diseases is slowly reaching a crisis point. There are resistant and multi-resistant pathogenic bacteria detected in wastewater, sewage treatment plants as well as in other environment sectors (Singh et al. 2019). Furthermore, in arid regions, wastewater containing antibiotic resistant bacteria is used for irrigation, and sewage sludge serves as fertilizers. Thus, this allows antibiotic resistant bacteria to enter the food chain directly (Singh et al. 2019). Hospital wastewater can be hazardous to public health and ecological balance. Many studies have demonstrated that wastewater from hospitals contribute to high rates of resistant bacteria that are being discharged in the natural environment. Waste effluent from hospitals contains adequate concentration of numerous resistant bacteria and antibiotic residues which inhibit the growth of susceptible bacteria. Hence, as a result, waste effluent of hospitals can also increase the numbers of resistant bacteria in the recipient sewers. In this work we analyzed the microbial composition of urban and rural wastewater which carries hospital waste (Singh et al. 2019) and found that severe disease causing pathogens are abundantly present having a number of antibiotic resistance pathways being overexpressed (Fig. 23.4). When enriched analyses were performed with the

Genera Enriched
Roseovarius; Burkholderia; Anaeromyxobacter
Rhodococcus; Paenibacillus;
Desulfovibrio
Arthrobacter; Acinetobacter; Pseudoalteromonas
Acinetobacter
Marmoricola
Flavobacterium
Lysobacter;Xanthomonas; Burkholderia;
Gemmata; Haliangium; Thioalkalivibrio;
Clostridium
Pseudomonas; Burkholderia;
Desulfovibrio; Desulfohalobium;
Desulfovibrio;
Flavobacterium;
Pseudoxanthomonas
Pseudomonas
Pseudoxanthomonas; Halomonas; Burkholderia;
Methylobacterium;Herbaspirillum
Mesorhizobium; Achromobacter; Stenotrophomonas;
Acinetobacter;
Desulfovibrio;
Desulfobulbus;Pseudomonas;

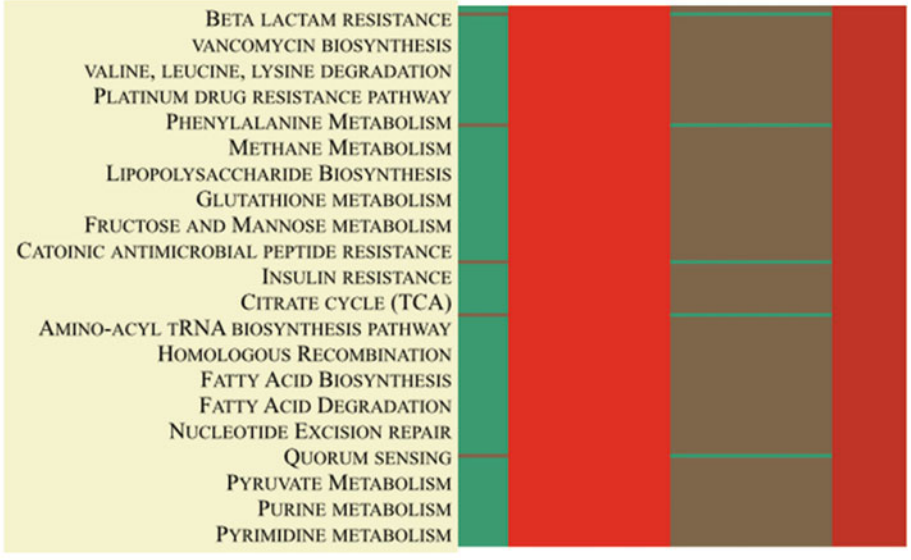


Fig. 23.2 Enriched genera and corresponding enriched pathways in the rhizospheric niche under study

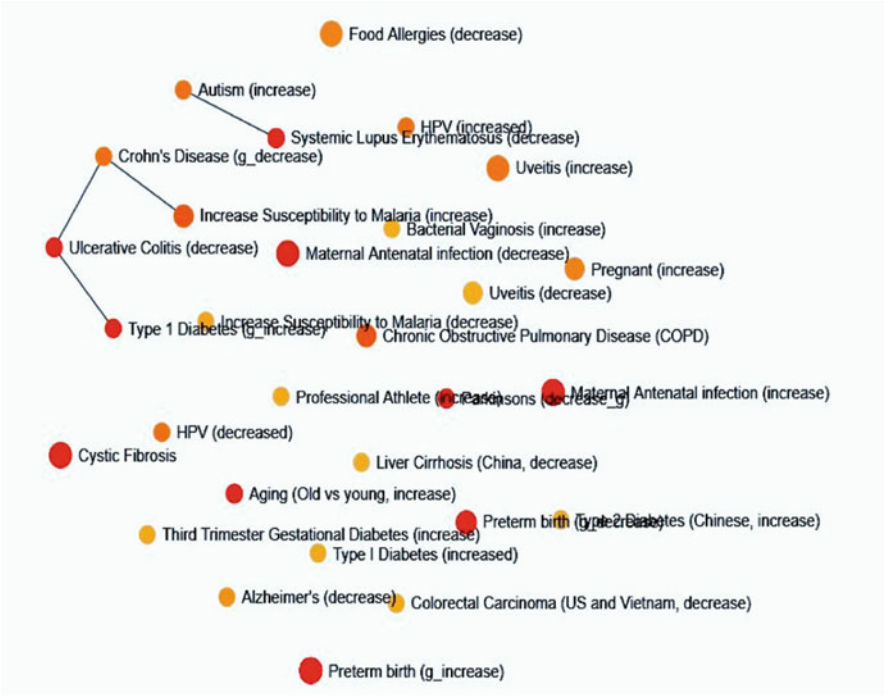


Fig. 23.3 Prediction of disease networks from rhizospheric microbial abundance

common microbes it was found that a complex disease network was prevalent in the area based on predictions (Fig. 23.5). This leads us to conclude that the variety of pathogenic organisms is much higher in both rural and urban wastewater samples, which are direct runoffs of hospital effluents thus increasing the inherent pathogenic load of the area.

23.5.5 Case Study 5

There are trillions of diverse bacteria which inhabit in human gastrointestinal tract and vary among individuals within and between communities. The initial inoculum of bacterium is acquired maternally during birth or inside womb. Subsequently, colonization of bacteria inside human gut depends upon several factors including diet, age, and diseases. The bacterial communities isolated from gastrointestinal exert phenotypic traits of the host by a complex network of interactions among them. Many of such interactions arising from modified gut bacterial profiles (GBP) have been observed to cause diseases in human. Moreover, modern lifestyle of the western countries makes people more prone to inflammatory disorders with altered GBP. The GBP of several population of the world, both with modern and traditional lifestyles have been studied from America, Europe, Africa, Korea, and China. The

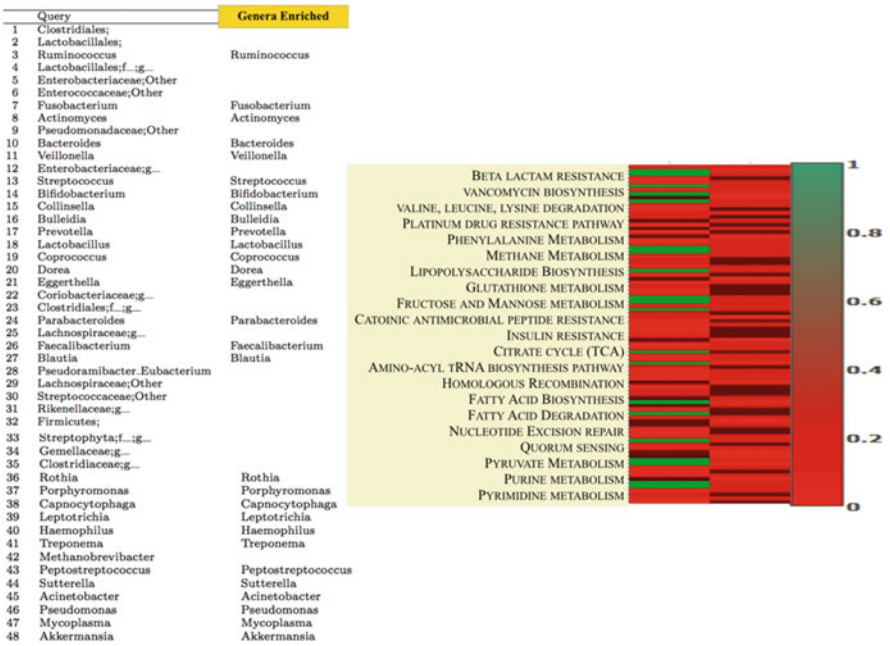


Fig. 23.4 Enriched genera and corresponding enriched pathways in the wastewater niche under study

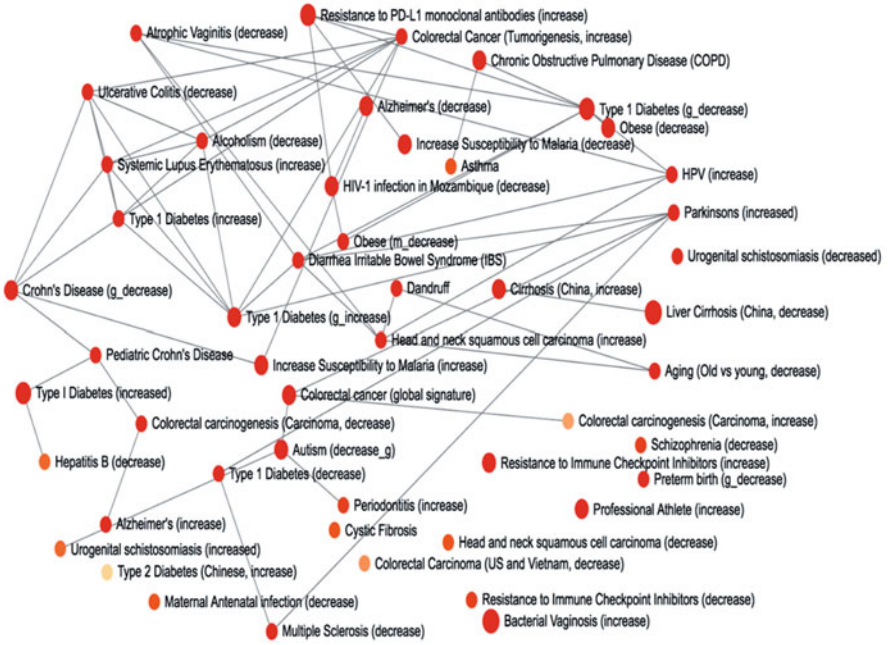


Fig. 23.5 Prediction of disease networks from microbial abundance dative the wastewater niche under study

	Query	Genera Enriched
1	unclassified (derived from Bacteria)	
2	Clostridium	Clostridium
3	unclassified (derived from Verrucomicrobia subdivision 3)	
4	Propionibacterium	Propionibacterium
5	unclassified (derived from Planctomycetaceae)	
6	Bacteroides	Bacteroides
7	Kineococcus	
8	Coptotermes	
9	Lactobacillus	Lactobacillus
10	Bifidobacterium	Bifidobacterium
11	Prevotella	Prevotella
12	Oryza	
13	Candidatus Solibacter	
14	Paenibacillus	Paenibacillus
15	Heliobacterium	
16	unclassified (derived from Alphaproteobacteria)	
17	unclassified (derived from unclassified sequences)	
18	Bacillus	Bacillus
19	Atopobium	Atopobium
20	Porphyromonas	Porphyromonas

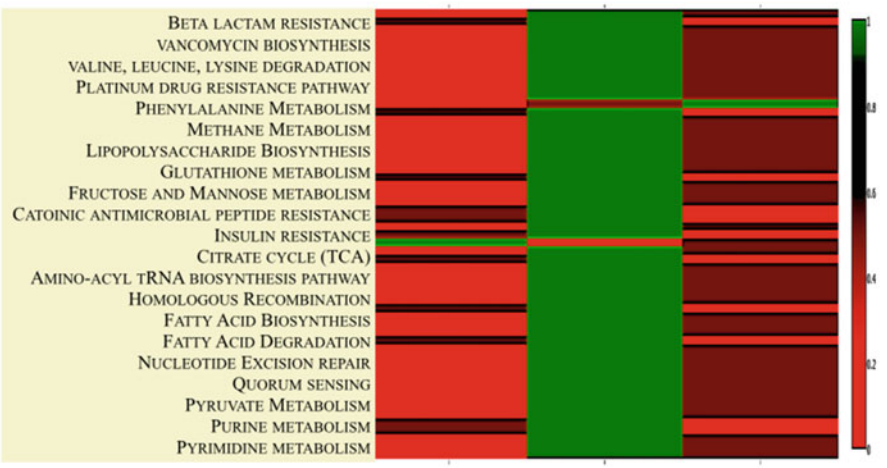


Fig. 23.6 Enriched genera and corresponding enriched pathways in the gut microbiomes under study

large tribal population of India offers a unique scenario for studies on gut bacterial profiles, because India consist of diverse communities who still depend on hunting, agriculture, and fishing along with their own culture, tradition, dietary habits, language, and genetic adaptability (Ganguli et al. 2019).

In this work fecal samples were collected from a tribal family belonging to the Dhrupka Bhutia tribal community and were subsequently sequenced using OXFORD Nanopore Minion sequencing platform for better elucidation of the bacterial members. Results obtained showed heterogenous abundance profiles of the bacterial members with the highest in case of male being *Lactobacillus*, for female: *Enterobacteria* and *Rothia* and for their male kid: *Leuconostoc* and *Fusobacterium*. Interesting observation was no antibiotic resistance pathway was identified in the pathway enrichment analysis (Fig. 23.6) which can be justified by the fact these tribal communities are not exposed to the over the counter medicines

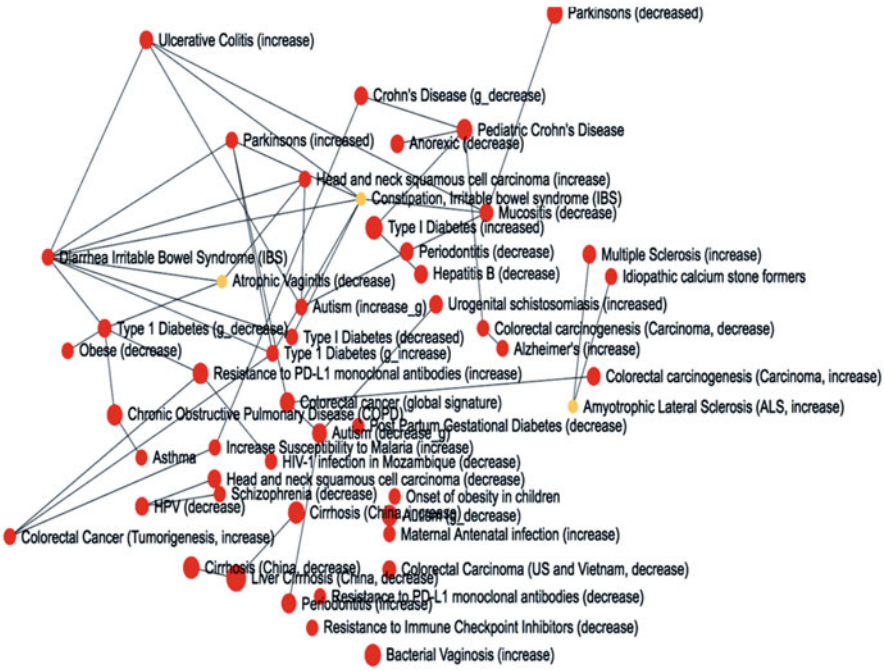


Fig. 23.7 Prediction of disease networks from microbial abundance data of the gut microbiomes under study

due to their remote habitat and extreme environmental conditions. Thus, their gut is still not exposed to antibiotic resistant bacteria, however, the presence of *Exiguo bacterium* in the gut is a clear warning to the threats of microplastics in the diet. Yang et al., have reported the ability of this bacterial strain to utilize plastics (Yang et al. 2014). It is thus alarming that this particular member has established itself as an important member of the gut of even tribal people whose gut is thought to be unadulterated and pristine. The disease network analysis revealed that irritable bowel syndrome and liver cirrhosis were important nodes (Fig. 23.7) which may be attributed to the inclination of these tribal members in having a regular dose of alcohol in their diet.

If we observe closely then all the predictions have their unique pathways which support the inter disease network analysis. While rhizospheric niche possesses microbes for food allergies as there may be several plant associations and exudates in the rhizosphere, hospital wastewater presents a complex biological niche laden with antimicrobial resistance pathways as well as behavioral disease pathways for diabetes, COPD, and vaginitis. The gut microbial datasets from remote tribes also exhibit characteristics features of no resistance pathways and lesser communicable disease pathways. These data further indicate the robustness of the pipeline in successful prediction of pathogenic load and possible disease prevalence in the

areas under study also providing a background insight on diet practices and medicine usage.

23.6 Conclusion

The above case studies indicate that culture-independent mechanism of metagenomics can be utilized properly for predicting the pathogenic load from a variety of samples from different environmental and disease associated niches. The DNA extraction mechanisms are vigorously standardized worldwide in equipped laboratories, where 21 DNA extraction protocols have been evaluated and reported recently. These protocols have contributed to comparison of microbial community and organization of DNA purification steps, with a conclusion that shows the largest outcome of preferably raw sequenced data and associated metadata, which is the ultimate focus for diagnostics. Following the abundance mapping and enrichment analyses steps a clear picture can be predicted which provides us with the necessary information on what pathogenic microbes may be present, what are the enriched biological pathways that are prevalent in the consortium and finally what disease associations can be prevalent. Once all technical and ethical barriers are overcome, we believe that metagenomic guided environment impact assessment, will be the next big area of research in the near future having the potential to alter the policymakers perspective on climate change and associated healthcare.

Acknowledgement The authors are indebted to the Department of Science and Technology, West Bengal for the financial support provided to Dr. Mahashweta Mitra Ghosh, Dr. Subrata Sankar Bagchi and Dr. Sayak Ganguli for carrying out the necessary sequencing of samples and for fellowship to Meesha Singh and Somosree Pal. The authors also acknowledge the support of the Principal, St. Xavier's College (Autonomous), Kolkata, Barasat Government College and Bangabasi College, Kolkata for providing the necessary infrastructure for carrying out the work.

References

Aarestrup FM, Koopmans MG (2016) Sharing data for global infectious disease surveillance and outbreak detection. Trends Microbiol 24(4):241–245

Albertsen M, Hugenholtz P, Skarshewski A, Nielsen KL, Tyson GW, Nielsen PH (2013) Genome sequences of rare, uncultured bacteria obtained by differential coverage binning of multiple metagenomes. Nat Biotechnol 31(6):533

Allard MW, Strain E, Melka D, Bunning K, Musser SM, Brown EW, Timme R (2016) Practical value of food pathogen traceability through building a whole-genome sequencing network and database. J Clin Microbiol 54(8):1975–1983

Alneberg J, Bjarnason BS, De Bruijn I, Schirmer M, Quick J, Ijaz UZ, Lahti L, Loman NJ, Andersson AF, Quince C (2014) Binning metagenomic contigs by coverage and composition. Nat Methods 11(11):1144–1146

Andersen SC, Fachmann MSR, Kiil K, Møller Nielsen E, Hoorfar J (2017a) Gene-based pathogen detection: can we use qPCR to predict the outcome of diagnostic Metagenomics? Genes 8 (11):332

Andersen SC, Kiil K, Harder CB, Josefsen MH, Persson S, Nielsen EM, Hoorfar J (2017b) Towards diagnostic metagenomics of campylobacter in fecal samples. BMC Microbiol 17(1):133

Cleary B, Brito IL, Huang K, Gevers D, Shea T, Young S, Alm EJ (2015) Detection of low-abundance bacterial strains in metagenomic datasets by eigengenome partitioning. Nat Biotechnol 33(10):1053

Ganguli S, Rahaman S, Bera AR, Vishal V, Malik S, Roopalakshmi K, Singh PK (2017) Rhizospheric metagenome of the terrestrial mangrove fern Acrostichum from Indian Sunderbans. Genomics Data 14:53–55

Ganguli S, Pal S, Das K, Banerjee R, Bagchi SS (2019) Gut microbial dataset of a foraging tribe from rural West Bengal - insights into unadulterated and transitional microbial abundance. Data Brief 25:103963. https://doi.org/10.1016/j.dib.2019.103963

Gilbert JA, Jansson JK, Knight R (2014) The earth microbiome project: successes and aspirations. BMC Biol 12(1):69

Haak W, Lazaridis I, Patterson N, Rohland N, Mallick S, Llamas B, Brandt G, Nordenfelt S, Harney E, Stewardson K, Fu Q (2015) Massive migration from the steppe was a source for indo-European languages in Europe. Nature 522(7555):207

Karsenti E, Acinas SG, Bork P, Bowler C, De Vargas C, Raes J, Sullivan M, Arendt D, Benzoni F, Claverie JM, Follows M (2011) A holistic approach to marine eco-systems biology. PLoS Biol 9 (10):e1001177

Key FM, Posth C, Krause J, Herbig A, Bos KI (2017) Mining metagenomic data sets for ancient DNA: recommended protocols for authentication. Trends Genet 33(8):508–520

Lindgreen S, Adair KL, Gardner PP (2016) An evaluation of the accuracy and speed of metagenome analysis tools. Sci Rep 6:19233

Loman NJ, Constantinidou C, Christner M, Rohde H, Chan JZM, Quick J, Weir JC, Quince C, Smith GP, Betley JR, Aepfelbacher M (2013) A culture-independent sequence-based metagenomics approach to the investigation of an outbreak of Shiga-toxigenic Escherichia coli O104: H4. JAMA 309(14):1502–1510

Mallick S, Li H, Lipson M, Mathieson I, Gymrek M, Racimo F, Zhao M, Chennagiri N, Nordenfelt S, Tandon A, Skoglund P (2016) The Simons genome diversity project: 300 genomes from 142 diverse populations. Nature 538(7624):201–206

Mande SS, Mohammed MH, Ghosh TS (2012) Classification of metagenomic sequences: methods and challenges. Brief Bioinform 13(6):669–681

Menzel P, Ng KL, Krogh A (2016) Fast and sensitive taxonomic classification for metagenomics with Kaiju. Nat Commun 7(1):1–9

Micah H, Claire FL, Rob K (2007) The human microbiome project: exploring the microbial part of ourselves in a changing world. Nature 449(7164):804–810

Miller RR, Montoya V, Gardy JL et al (2013) Metagenomics for pathogen detection in public health. Genome Med (5):81. https://doi.org/10.1186/gm485

Naccache SN, Federman S, Veeraraghavan N, Zaharia M, Lee D, Samayoa E, Bouquet J, Greninger AL, Luk KC, Enge B, Wadford DA (2014) A cloudcompatible bioinformatics pipeline for ultrarapid pathogen identification from next-generation sequencing of clinical samples. Genome Res 24(7):1180–1192

Nakamura S, Maeda N, Miron IM, Yoh M, Izutsu K, Kataoka C, Honda T, Yasunaga T, Nakaya T, Kawai J, Hayashizaki Y (2008) Metagenomic diagnosis of bacterial infections. Emerg Infect Dis 14(11):1784

Nielsen HB, Almeida M, Juncker AS, Rasmussen S, Li J, Sunagawa S, Plichta DR, Gautier L, Pedersen AG, Le Chatelier E, Pelletier E (2014) Identification and assembly of genomes and genetic elements in complex metagenomic samples without using reference genomes. Nat Biotechnol 32(8):822–828

Orlando L, Ginolhac A, Zhang G, Froese D, Albrechtsen A, Stiller M, Schubert M, Cappellini E, Petersen B, Moltke I, Johnson PL (2013) Recalibrating Equus evolution using the genome sequence of an early middle Pleistocene horse. Nature 499(7456):74–78

Ounit R, Wanamaker S, Close TJ, Lonardi S (2015) CLARK: fast and accurate classification of metagenomic and genomic sequences using discriminative k-mers. BMC Genomics 16(1):236
Pallen MJ (2014) Diagnostic metagenomics: potential applications to bacterial, viral and parasitic infections. Parasitology 141(14):1856–1862
Petersen TN, Lukjancenko O, Thomsen MCF, Sperotto MM, Lund O, Aarestrup FM, Sicheritz-Pontén T (2017) MGmapper: reference based mapping and taxonomy annotation of metagenomics sequence reads. PLoS One 12(5):e0179778
Rahaman S, Bera AR, Vishal V, Gupta S, Chakraborty W, Ganguli S (2019) Biochemical and metagenomic insight into the impact of climate change on an epiphytic fern of the Indian Sunderbans. Trop Plant Res 6(3):433–437
Ranjan R, Rani A, Metwally A, McGee HS, Perkins DL (2016) Analysis of the microbiome: advantages of whole genome shotgun versus 16S amplicon sequencing. Biochem Biophys Res Commun 469(4):967–977
Rodriguez-r LM, Konstantinidis KT (2014) Estimating coverage in metagenomic data sets and why it matters. ISME J 8(11):2349–2351
Sangwan N, Xia F, Gilbert JA (2016) Recovering complete and draft population genomes from metagenome datasets. Microbiome 4(1):8
Schlebusch CM, Skoglund P, Sjödin P, Gattepaille LM, Hernandez D, Jay F, Li S, De Jongh M, Singleton A, Blum MG, Soodyall H (2012) Genomic variation in seven Khoe-san groups reveals adaptation and complex African history. Science 338(6105):374–379
Schneeberger PH, Becker SL, Pothier JF, Duffy B, N'Goran EK, Beuret C, Frey JE, Utzinger J (2016) Metagenomic diagnostics for the simultaneous detection of multiple pathogens in human stool specimens from Cote d'Ivoire: a proof-of-concept study. Infect Genet Evol 40:389–397
Sedlar K, Kupkova K, Provaznik I (2017) Bioinformatics strategies for taxonomy independent binning and visualization of sequences in shotgun metagenomics. Comput Struct Biotechnol J 15:48–55
Segata N, Boernigen D, Tickle TL, Morgan XC, Garrett WS, Huttenhower C (2013) Computational meta'omics for microbial community studies. Mol Syst Biol 9(1):666
Segata N, Waldron L, Ballarini A, Narasimhan V, Jousson O, Huttenhower C (2012) Metagenomic microbial community profiling using unique clade-specific marker genes. Nat Methods 9(8):811
Singh M, Ganguli S, Ghosh MM (2019) Comparative metagenomic dataset of hospital effluent microbiome from rural and urban hospitals in West Bengal. Data Brief 25:104264
Skoglund P, Thompson JC, Prendergast ME, Mittnik A, Sirak K, Hajdinjak M, Salie T, Rohland N, Mallick S, Peltzer A, Heinze A (2017) Reconstructing prehistoric African population structure. Cell 171(1):59–71
Warinner C, Herbig A, Mann A, Fellows Yates JA, Weiß CL, Burbano HA, Orlando L, Krause J (2017) A robust framework for microbial archaeology. Annu Rev Genomics Hum Genet 18:321–356
Wood DE, Salzberg SL (2014) Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome Biol 15(3):R46
Yun S, Yun S (2014) Masking as an effective quality control method for next-generation sequencing data analysis. BMC Bioinform 15(1):382
Yang J, Yang Y, Wu WM, Zhao J, Jiang L (2014) Evidence of polyethylene biodegradation by bacterial strains from the guts of plastic-eating waxworms. Environ Sci Technol 48 (23):13776–13784

Chapter 5
Metagenomics-Guided Assessment of Water Quality and Predicting Pathogenic Load

Sayak Ganguli
https://orcid.org/0000-0001-5481-7436
St. Xavier's College (Autonomous), Kolkata, India

Rupsha Karmakar
Vivekananda College for Women, India

Meesha Singh
St. Xavier's College (Autonomous), Kolkata, India

Mahashweta Mitra Ghosh
St. Xavier's College (Autonomous), Kolkata, India

ABSTRACT

Antibiotic-resistant bacteria (ARB) are becoming more prevalent in the environment and are efficiently disseminating through contaminated wastewater resulting in resistome cycling. This chapter compares the bacterial profile of hospital effluents collected from rural, urban, and delta regions of West Bengal, India. Comparative metagenomics analysis identified pathogenic bacterial genera like pseudomonas, escherichia, staphylococcus, lactobacillus, prevotella, acinetobacter across the samples. Delta sample showed highest abundance of pseudomonas whereas rural sample had lower titre of all the common bacterial genera. Urban sample reflected more diversity of different genera in terms of abundance. Pathogenic load prediction revealed significant occurrence of diarrhea, irritable bowel syndrome, liver cirrhosis, ulcerative colitis in the disease network. This chapter proposes a monitoring programme for assessing wastewater health using a combination of culture independent and culture-dependent molecular techniques in order to prevent the spread of pollutants in tropical environments.

INTRODUCTION

DOI: 10.4018/978-1-7998-9498-8.ch005

A Well-developed sewage infrastructure, efficient wastewater treatment, and availability of drinking water are just a few of the basic requirements for maintaining and improving the public health (Bürgmann et al., 2018). Water management systems around the world are confronted with a huge issue of contamination of water supplies. One of the few reasons behind this contamination is lack of proper sanitary infrastructure which pollutes the environment and pervades all societal processes leading to human health risks that in turn can reduce overall developmental potential. According to the United Nations, about 80 percent of sewage is discharged in the adjoining aquatic bodies without any treatment (UN WWAP (United Nations World Water Assessment Programme), 2017). Animal excreta, variety of biomolecules, decomposition products, sands, grits, natural and synthetic organic substances from industrial sources, various micronutrients, macronutrients, heavy metals, traces of antibiotics are the basic contents of domestic, industrial and hospital effluents (Holmberg, 2019; Ajiboye et al., 2021; Hassan et al., 2020). Hospital wastes are in constant limelight as these include antibiotic residues such as Oxytetracycline, Tetracycline, Ciprofloxacin, Fluoroquinolones, Sulfonamides, Macrolides, Trimethoprim, disinfectants, anaesthetics, lipid regulators, radioactive markers, organic matters, excreta from patients and many more (Hassan et al., 2020; Ngigi et al., 2019; Thai et al., 2018). Additionally, various kinds of pathogenic microbial strains can be found in wastewater (Yasir, 2021).

Moreover, domestic sewage, hospital effluents and drainage from animal husbandry act as reservoirs of Antibiotic resistant bacteria (ARB) and Antibiotic resistance genes (ARGs) (He et al., 2020; Khan et al., 2019). ARBs have increased their population in the environment due to severe use of antibiotics for medical purposes and their continuous release into the environment (Serwecińska, 2020; Kraemer et al., 2019). World Health Organization has classified ARGs as a new contaminant due to their increasing incidence and widespread distribution. Extensive use of antibiotics in medicine and agriculture has been recognized as one of the major causes of rising ARGs. Similarly, heavy metal exposure from various sources can influence the spread of Metal resistant genes (MRGs) and ARGs. Heavy metal resistance and antibiotic resistance can be selected simultaneously in the heavy metal contamination environment. Two mechanistic interpretations of this event are co-selection (selection of two or more genetically linked resistant genes) or cross-selection (an event where a single genetic element offers tolerance to many antimicrobial drugs) (Chen et al., 2019). In agriculture, animal husbandry, wastewater treatment systems and sediment, the co-selection of MRGs and ARGs has been documented (Wang et al., 2021; Li et al., 2021; Yuan et al., 2018). Bacterial communities, on the other hand, are influenced by a diverse set of evolutionary, ecological, and environmental factors which includes the concentration and distribution of MRGs and ARGs also (Chen et al., 2019). Studies have proven that various constituents of waste water, such as antibiotic residues, disinfectants, and heavy metals exert selection pressure for antibiotic resistance (Karkman et al., 2018). Several studies have recently revealed that antibiotic residues, resistant bacteria, and resistance genes are well abundant in the clinical sewages as well as in the recipient aquatic ecosystems which clearly indicates the sites of the development of antibiotic resistance in the environment (Voigt et al., 2020). Being a hotspot of drug resistant organisms, hospital wastes elevate the numbers of resistant bacteria in the recipient sewers facilitating the resistant gene transfer in the bacterial strain through recombination, horizontal gene transfer, mutation etc (Mondal et al., 2021; Abe et al., 2020). In due course of time these Antibiotic Resistant bacteria get the chance to enter the food chain when wastewaters are used for irrigation and sewage sludges are applied as fertilizer (Lamastra et al., 2018). Untreated human sewage reaches streams by point source discharges or combined sewer overflows from wastewater treatment plants (Mansfeldt et al., 2020; Quintela-Baluja et al., 2019). These

streams are in turn utilized to provide drinking water to the community. Wastewater treatment plants (WWTPs) have been proved to curtail the release of antibiotics, pathogenic microorganisms into the environment successfully (Aghalari et al., 2020). But the efficiency is only 10 to 100 folds in terms of microbial reduction (Karkman et al., 2018). Multiple studies have revealed the presence of several antibiotic resistance genes (ARGs) and metal resistance genes (MRGs) from metagenomes collected from influent, activated sludge (AS) and digested sludge (DS) of Urban and Municipal WWTPs (Wang et al., 2020; Sun et al., 2021; Yoo et al., 2020). The above claims can be correlated through the widespread use of antibiotics in human and veterinary medicine resulting in antibiotic resistance and spreading it across the wastewater network. A study in Göttingen, Germany showed dominance of pathogenic bacteria in the samples collected from hospital effluent at different steps of wastewater treatment (Schneider et al., 2020). Hence, discharge of treated effluents is equally responsible for contaminating adjoining natural water bodies with ARGs and MRGs.

The emergence of novel antibiotic-resistant bacterial strains that are resistant to the majority of currently available antimicrobial drugs has become a serious challenge in modern medicine, especially with the rise in immune compromised patients. They are vigorously harming public health with economic and social consequences around the world. According to the World Health Organization, diseases like diarrhoea, cholera, dysentery, typhoid can all be spread by contaminated water. As per WHO, each year, 4,85,000 diarrhoeal fatalities are estimated to be caused by contaminated drinking water. Antibiotic resistance chart shows increasing resistance of pathogens toward the antibiotics like Amikacin, Carbapenems, aminoglycosides, Cephalosporins etc. From 2008 to 2020, consistent increase in the resistance events is noticeable (The Center for Disease, 2021). A report from the European Medicines Agency and the European Centre for Disease Prevention and Control has revealed the death of about 25000 patients annually in the European Union from an infection with the Multi Drug Resistant (MDR) bacteria. They have warned that very few antibacterial agents are left to deal with this drug resistant catastrophes. For decades no unique mechanism of action and novel target agents are in the trail to deal with the situation (European Medicines Agency and the European Centre for Disease Prevention and Control, 2009). A list of Global Priority Pathogens (GPP) containing 12 species of bacteria categorized under 3 priority ranks was published by World Health Organization (WHO) in 2017 with the objective to prioritize the need of new therapeutic agents (WHO, 2017).

BACKGROUND

Characterization of wastewater microbial composition and potential pathogenic load is mandatory to predict the overexpressed resistance pathways along with prevalent disease network. Wastewater-based epidemiology (WBE) that means wastewater screening as a tool of public health monitoring is now widely accepted (Daughton, 2018; Xagoraraki & O'Brien, 2020). WBE entails collection of wastewaters, analysis of drug or metabolite residues in wastewater, computational outputs of drug consumption and drug excretion rates which enables to estimate drug prevalence and consumption by a particular population. It has the advantages of being less costly and producing more reliable, near-real-time data. It augments a lot of promise as a supplement to current drug monitoring systems. Wastewater-based epidemiology can be used to create large-scale monitoring networks that reveal spatial patterns and temporal trends in drug misuse (Feng et al., 2018). For the last few years WBE have been used to treat a diverse of water-

borne, foodborne, faecal-oral pathogenic bacteria and viruses, which are usually excreted through the stool of the infected people (Bisseux et al., 2018). Frequent analysis of municipal wastewater helps to evaluate the viral circulation pattern in the population as they act as ideal sampling station concentrating discharges from the entire community.

Sudden emergence of COVID-19 pandemic has forced the world to reconsider the matter of wastewater hazards. Multiple studies have reported the traces of SARS-CoV-2 coronavirus in municipal wastes (Ahmed et al., 2020a), hospital sewage (Zhang et al., 2020), activated sludges derived from wastewater treatment plants (Kocamemi et al., 2020) and even in the river water (Rimoldi et al., 2020). Usually, excreta of patients which are expected to run away in sewers systems eventually ends up into wastewater and sewage. Thus, sanitary plumbing system is considered to be the prime route of viral transmission from community into the water and wastewater system (Ahmed et al., 2020a; Barcelo, 2020). Besides from faeces and urine of the patients, casual disposal of surgical face masks and medical equipment containing viral traces is another way that add viral load in sewer systems (Chin et al., 2020). Guerrero-Latorre et al., 2020 and Rimoldi et al., 2020 reported the existence of SARS-CoV-2 virus in the river water of Ecuador and Italy respectively which receives effluents from all over the city. Rosa et al. (2020) collected multiple sewage samples from various sites of Milan and Rome, Italy between the time span of 3rd February 2020 to 2nd April 2020. Molecular analysis of samples confirmed the occurrence of SARS-CoV-2 RNA fragments in the test samples. Most interesting point to be noted is that the first Italian SARS-CoV-2 positive case was reported on February 21st. Significantly, COVID-19 infections were still rare in Italy by the end week of February 2020, when the sludge samples were found to be positive for SARS-CoV-2 in Milan. These sorts of findings demonstrate the sensitivity of environmental surveillance in detecting continuing pandemics in the general population. Viral identification in sewage, despite of the low prevalence of reported human illnesses, may be linked to sewage surveillance's capacity to estimate mild or asymptomatic cases following rigorous epidemiological modelling. Here lies the success of Wastewater-based epidemiology (WBE). The current pandemic can pose a long-term influence on the event of antimicrobial resistance (AMR), causing an additional burden to the environment. Antibiotics are given to up to 70% of COVID-19 patients in either an outpatient or hospital setting (Langford et al., 2021). Overuse of antimicrobial agents boosts the emergence of AMR in co-infecting pathogens (Tedijanto et al., 2018). Thus, this pandemic may place an even greater focus on the importance of wastewater monitoring.

Various crucial information about human health-related aspects such as pathogen distribution, potential disease threat, occurrence of antibiotic resistance genes can be well characterized through Metagenomics analysis of wastewater samples as a part of diagnostic metagenomics (Yoo et al., 2020; Breitwieser & Salzberg, 2020). Diagnostic metagenomics has the capacity to optimally trace all the pathogenic microbes as well as the diseases induced by corresponding microbes in a variety of samples such as faeces, urine, blood and even wastewater (Greninger, 2018). For instances, several tick-borne disease pathogens like *Anaplasma, Ehrlichia*, *Borrelia* species has been well identified using targeted metagenomic sequencing (Thoendel, 2020). In general microbes inhabit almost every niche on the earth, demonstrating the immense diversity of the microbial world. Many of them have been documented to be significant members of their own ecosystems playing key roles in a variety of environmental and host-related biological processes. Majority of these microbes are still unexplored and their impacts in the respective habitats are unknown due to their unculturability. The properties like poor growth rates, transitions into dormancy prevent the unculturable bacteria to grow in typical laboratory media (Bodor et al., 2020). Metagenomics, a modern

culture-independent method for studying microbial community in various habitats, has opened up new paths for resolving common microbiological issues such as documentation of total microbial species composition in a given environment, assessment of their ecological impacts and many more (Datta et al., 2020). Metagenomics study of environmental DNA that is genetic material extracted from environmental samples can be used to improve public health. For example, environmental DNA can be used to assess the diversity of microbes and to track probable contamination sources in water bodies (Staley et al., 2018). DNA or RNA sequencing using next-generation sequencers, sequence assembly, gene prediction, functional and metabolic analysis, taxonomic binning and comparative analysis of the sequence data using specialized bioinformatics tools are the basic steps involved in a typical metagenomic project to estimate the number of species and the functional repertoire of an environment. Whereas, inherent difficulties with the metagenomic data, such as incomplete coverage, huge volumes of raw sequence data produced by next-generation sequencers, and short lengths of the sequence reads make each stage of the analysis more challenging (Zou et al., 2018). Metagenomics is an excellent approach to monitor AMR in sewage system. For thorough and accurate profiling of antimicrobial resistant genes, metagenomic data relies on high throughput sequencing of all community DNA and sequence depth. Global monitoring of untreated urban sewage through metagenomic study reported a significant abundance of ARGs conferring resistance to beta-lactams, tetracycline, macrolides, and aminoglycosides (Hendriksen et al., 2019). Kutilova et al., 2021 has conducted a metagenomic study of wastewater community DNA obtained from hospital and municipal waste effluent. High titer of pathogenic members of bacterial genera like *Pseudomonas, Escherichia, Klebsiella, Aeromonas, Enterobacter and Arcobacter* were noted in the samples. Hendriksen et al., 2019 has recommended metagenomic analysis of municipal sewage as a morally acceptable and cost-effective method for infectious disease surveillance and AMR prediction. Hence during the event of a public health emergency use of metagenomics databases allows prediction of potential disease threats in advance.

SUGGESTED PROTOCOL FOR WASTEWATER MONITORING

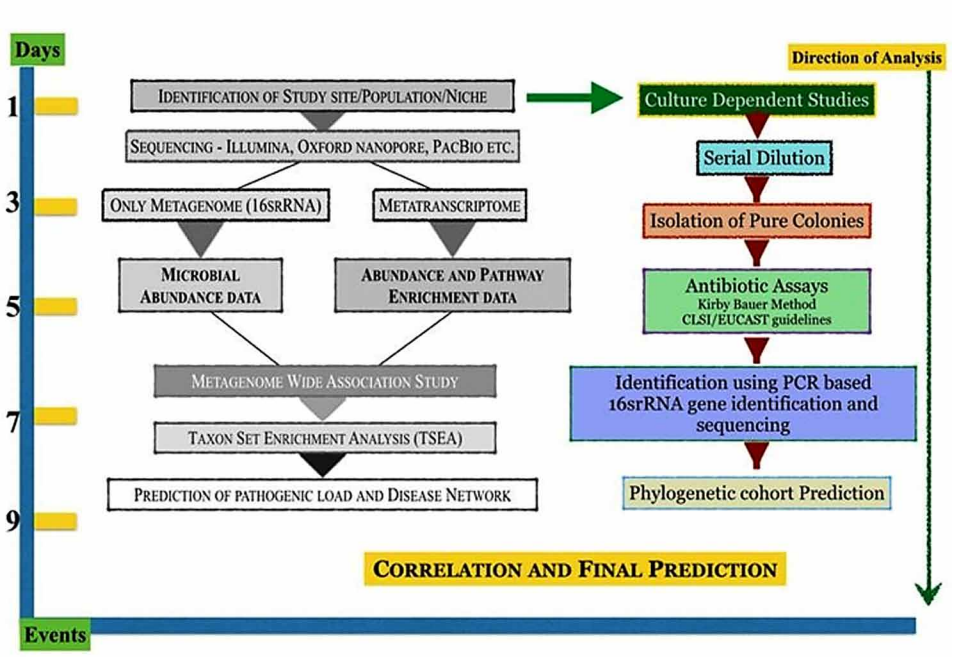
A comparative protocol has been formulated which utilizes both metagenomics guided data acquisition along with validation by standard culture dependent mechanisms.

From the metagenomics-based analysis, generation of a predicted pathogenic load network graph is possible which takes into account taxon enrichment and also enables to correlate the data with genus abundance profiles. The culture dependent segment can be used to validate and quantify the extent of antibiotic resistance in the samples under study. The detailed protocols are listed in Figure 1.

SAMPLE COLLECTION

Sludge samples need to be collected from a suitable collection site that accumulates community wastes. Followed by collection, samples should be kept in sterile containers at -20°C until they are ready to be processed. Subsequent culture based and culture independent steps need to be initiated within 10 hours of sample collection to obtain proper reproducible results. Stock samples can be stored at 4°C for future study.

Figure 1. Proposed pipeline for wastewater monitoring



Protocol for Metagenomic Sequencing

Further processing of the raw samples should be initiated from DNA isolation following the protocol by Bonet et al., 2012. Followed by isolation, DNA quantification should be performed using Qubit dsDNA HS Assay kit (Life Tech). Then, Nextera XT Idex Kit (Illumina, Inc.) need to be employed in the 16S Metagenomic Sequencing Library preparation of amplicons. In the upcoming step, V3 and V4 hypervariable regions of 16S rDNA gene of bacteria and Archaea need to be targeted for primer preparation. Next task should be the amplification and purification of amplicons using Illumina adaptors and 1 × AMpureXP beads respectively. The purified amplicons ought to be checked and quantified in the successive steps. In the final step, sequencing of the libraries following Illumina sequencing chemistry must be performed (Singh et al., 2019).

Computational Analysis of Metagenomic Data

Thorough profiling of microbial community should be initiated from taxon calling and OTU clustering of the processed paired ended reads using suitable tool that can assign properties to the individual sequence reads. After obtaining the abundance data, set of unique genera across the test samples need to be identified using an appropriate interactive tool. Followed by identification, abundance data of common bacterial genera should be easily visualized through heatmap. Heatmap implementation function of RStudio user interface called pheatmap() are able to make the step easier (IDE; RStudio Team, 2020; Kolde, 2019). Metagenome-wide association studies, linking the microbes of sample to host health and diseases ought to the next mandatory step. Hence, pathogenic load and related disease networks of the microbial members must be predicted in the final step through Taxon Set Enrichment Analysis (TSEA) with respect to a pre-defined taxon set, sharing similar traits (Dhariwal et al., 2017).

Culture Dependent Studies

Microbiological Characterization

Microbiological culturing of the collected effluent samples should be initiated from serial dilution and plating onto nutrient agar plates (pH 6.6) followed by overnight incubation at 37°C. Subculturing of each plates containing isolated colonies in increasing dilution provides pure colonies. Finally, under 100x magnification of the objective lens of a Bright Field Light Microscope, morphological inspection of the subcultured colonies can be performed, followed by Gram staining. Gram positive and Gram-negative bacterial isolates can be presumably identified using a series of traditional morphological, cultural, and biochemical assays, as outlined in Bergey's Manual of Determinative Bacteriology.

Biochemical Characterization

Biochemical assays for Catalase, Oxidase, Nitrate reduction, Indole, Methyl red, Voges- Proskauer, Citrate utilization, Urease, Starch hydrolysis, and Carbohydrate fermentation are all useful for characterization of the isolates (Cappuccino & Sherman, 2005).

Antibiotic Assay

The Kirby-Bauer disc diffusion assay can assess the susceptibility of isolates to various antibiotics. The choice of antibiotics for bacterial susceptibility testing should be based on CLSI 2019 recommendations. Antibiotic discs from HiMedia Laboratories are recommended to use in the disc diffusion experiments (Mumbai, India). Sensitivity to antibiotics (intermediate or susceptible) can be calculated on the basis of the information provided in manufacturer's manual followed by result interpretation using CLSI guidelines. Isolate showing resistance to three or more types of antibiotics ought to be classified as Multi Drug Resistant (MDR).

Bacterial Identification Using PCR Based 16s rRNA Gene Identification and Sequencing

The very first step of bacterial identification involves genomic DNA isolation from bacterial isolates. Second step is amplification and sequencing of 16S rDNA fragment using universal 16S primers 27f(5′AGAGTTTGATCCTGGCTCAG3′) and 1492r (5′TACGGTTACCTTGTTACGACTT3′) (Gerhardt et al. 1994). In the third step, raw sequences are put together using Cap3 Contig Assembly –in silico programme. In the last step, all the obtained sequences can be utilized to identify the bacterium through BLASTn tool (https://www.ncbi.nlm.nih.gov/).

Phylogenetic Cohort Prediction

Construction of the phylogenetic tree is aided by Clustal omega (Sievers et al., 2011) and Muscle (Edgar, 2004) which perform multiple sequence alignment for a fast validation of sequence scores. The phylogenetic tree module of the CLC Genomics workbench can determine the phylogenetic tree based on the consensus multiple sequence alignment (Felsenstein, 1985) with 100 bootstrap repetitions. Both

neighbour joining-based approaches (Saitou & Nei, 1987) and maximum likelihood-based methods (Felsenstein, 1981) must be used to evaluate the tree formed. Ultimately the best consensus tree should be chosen for analysis and interpretation. Discrimination of distinct clades using colour codes is possible by visualising the tree file in FigTree.

The efficiency of the protocol lies in the minimum time frame that is required to generate a plausible prediction in regard to water quality of collected samples. From our estimate this time frame is of 9 working days from the time of collection to the time of prediction of results (Figure 1).

SAMPLE DATA

Contamination of natural water with untreated sewage containing antibiotic residues, heavy metals, organic and inorganic chemicals exert a selective pressure for the rise of antibiotic resistant microorganisms posing a detrimental effect on human health. This in turn creates a socioeconomic burden on the community by directly reducing the therapeutic potency of widely used antibiotics and ultimately increasing community health care expenses (Wilson et al., 2020). Therefore, efficient management of clinical and household wastes has become a worldwide priority in recent years. Furthermore, as part of wastewater-based epidemiology (WBE), wastewaters and medical wastes are being analyzed on a regular basis to foretell the circulation pattern of pathogenic microbes as well as to predict possible disease network.

Keeping the success of WBE in mind, this particular study aims to compare the microbial community profile of wastewaters collected from Rural, Urban and Delta region in and around West Bengal, India. Rural sample represented the wastewater effluent collected from a medical facility located in Purulia. Whereas solid sludge and associated wastewaters obtained from a large medical facility in Central Kolkata was nominated as Urban sample. Effluent collected from the sewer system of a health care unit near the Deltaic region of Sundarbans was the Delta sample of this study. All the 3 samples were monitored using our proposed protocol. Metagenomics guided assessment provided a clear insight on abundances and pathogenic load of bacteria present in the collected sludge samples. An effort was made to predict possible disease network based on pathogenic load of the wastewater microbial community.

Comparison of wastewater microbial profile revealed that 19 bacterial genera were common throughout the Rural, Urban, and Delta samples under study. Noticeable common pathogenic genera were found to be *Pseudomonas, Escherichia, Staphylococcus, Lactobacillus, Prevotella, Acinetobacter* etc. Abundances of the respective bacterial genera have been represented through Table 1. Table 1 shows that *Cloacibacterium, Geobacter* and *Pseudomonas* are the highest abundant genera in Urban, Rural and Delta sample respectively. Common abundance data visualization through heatmap discloses that among the all genera, *Pseudomonas* exhibits highest abundance in Delta sample only. On the other hand, Urban sample shows much more diversity of different genera in terms of abundances compared to Rural and Delta sample. Surprisingly, all 19 common bacterial genera had lower titer of abundances in Rural sludge (Figure 2). Pathogenic load determination using taxon set enrichment analysis (TSEA) revealed higher probability of occurrence of multiple diseases in a network (Figure 3). Based on the disease network, Table 2 summarizes major diseases caused by drinking water contamination as a result of seepage of wastewater, such as diarrhoea, irritable bowel syndrome (IBS), liver cirrhosis, and ulcerative colitis, as well as their causative pathogens that are found in the samples. *Lactobacillus* is found to cause 3 major diseases such as Diarrhoea, Liver Cirrhosis and IBS. Liver Cirrhosis is also caused by bacterial genera *Prevotella* and *Streptococcus. Escherichia* and Streptococcus shows the potential to cause Ulcerative Colitis. Diseases

Table 1. Abundances of common microbial genera across the wastewater samples

Genus	Urban (%)	Rural (%)	Delta (%)
Cloacibacterium	5.22	0.06	0.17
Prevotella	2.85	0.36	0.13
Novosphingobium	2.02	0.11	0.10
Clostridium	1.44	0.24	0.01
Burkholderia	1.38	0.09	0.01
Acinetobacter	1.27	0.05	0.10
Escherichia	0.60	0.19	0.05
Streptococcus	0.30	0.23	0.02
Lactobacillus	0.30	0.17	0.09
Pseudomonas	0.29	0.07	2.40
Rhizobium	0.17	0.03	0.16
Sphingomonas	0.13	0.06	0.22
Acidovorax	0.11	0.13	0.01
Corynebacterium	0.10	0.05	0.01
Propionibacterium	0.10	0.18	0.12
Thauera	0.07	0.15	0.02
Staphylococcus	0.02	0.02	0.03
Rothia	0.02	0.02	0.17
Geobacter	0.02	0.38	0.06

Table 2. Noticeable wastewater related diseases and the causative pathogens obtained from predicted disease network of the common microbial genera across the wastewater samples

Disease	Associated Organisms	References
Diarrhea	*Lactobacillus*	(Agamennone et al., 2018)
Irritable Bowel Syndrome (IBS)	*Lactobacillus*	(Sadrin et al., 2020)
Liver Cirrhosis	*Lactobacillus, Prevotella, Streptococcus*	(Zhong et al., 2021)
Cirrhosis	*Streptococcus, Prevotella, Lactobacillus*	(Wang et al., 2019)
Ulcerative Colitis	*Escherichia, Streptococcus*	(Yang et al., 2020)
Chronic Obstructive Pulmonary Disease (COPD)	*Prevotella, Pseudomonas, Burkholderia, Lactobacillus*	(Deshpande et al., 2020)
Type I Diabetes	*Escherichia, Lactobacillus, Streptococcus*	(Etjahed et al., 2020)
Multiple Sclerosis	*Prevotella*	(Mirza et al., 2020)

like Chronic Obstructive Pulmonary Disease (COPD), Type 1 Diabetes, Multiple Sclerosis arises due to the secondary infections caused by *Prevotella, Pseudomonas, Burkholderia, Lactobacillus, Escherichia, Streptococcus* (Agamennone et al., 2018; Sadrin et al., 2020; Zhong et al., 2021; Wang et al., 2019; Yang et al., 2020; Deshpande et al., 2020; Etjahed et al., 2020; Mirza et al., 2020)

Figure 2. Heatmap representation of abundance data of the common genera

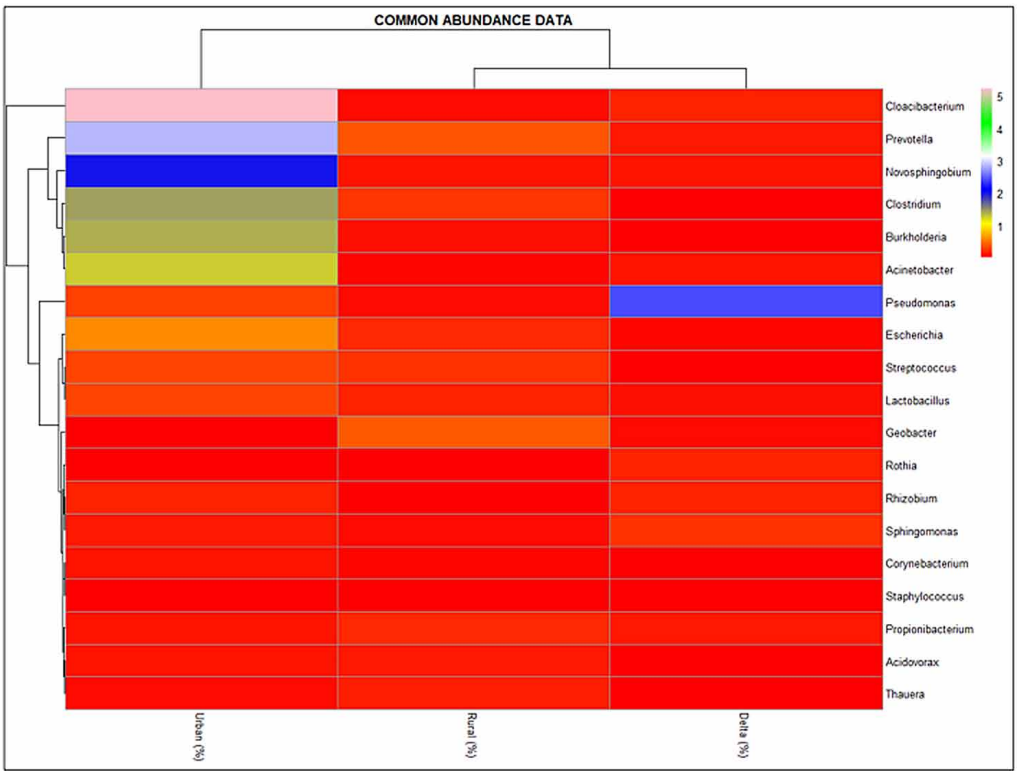
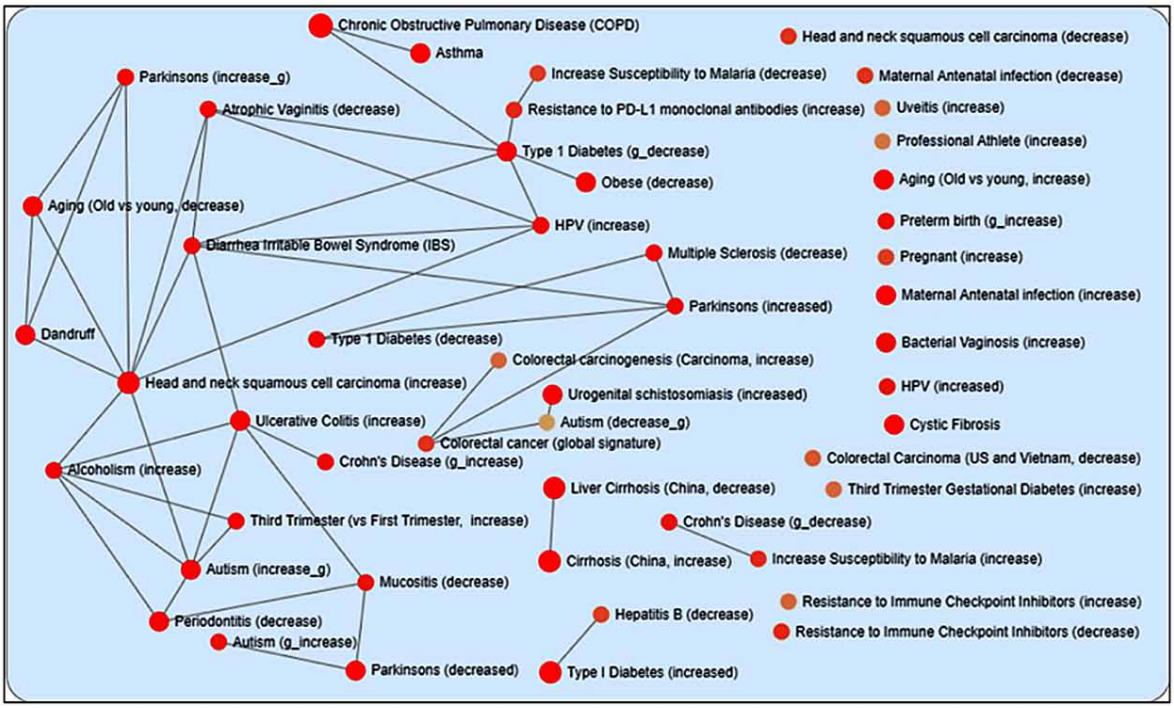


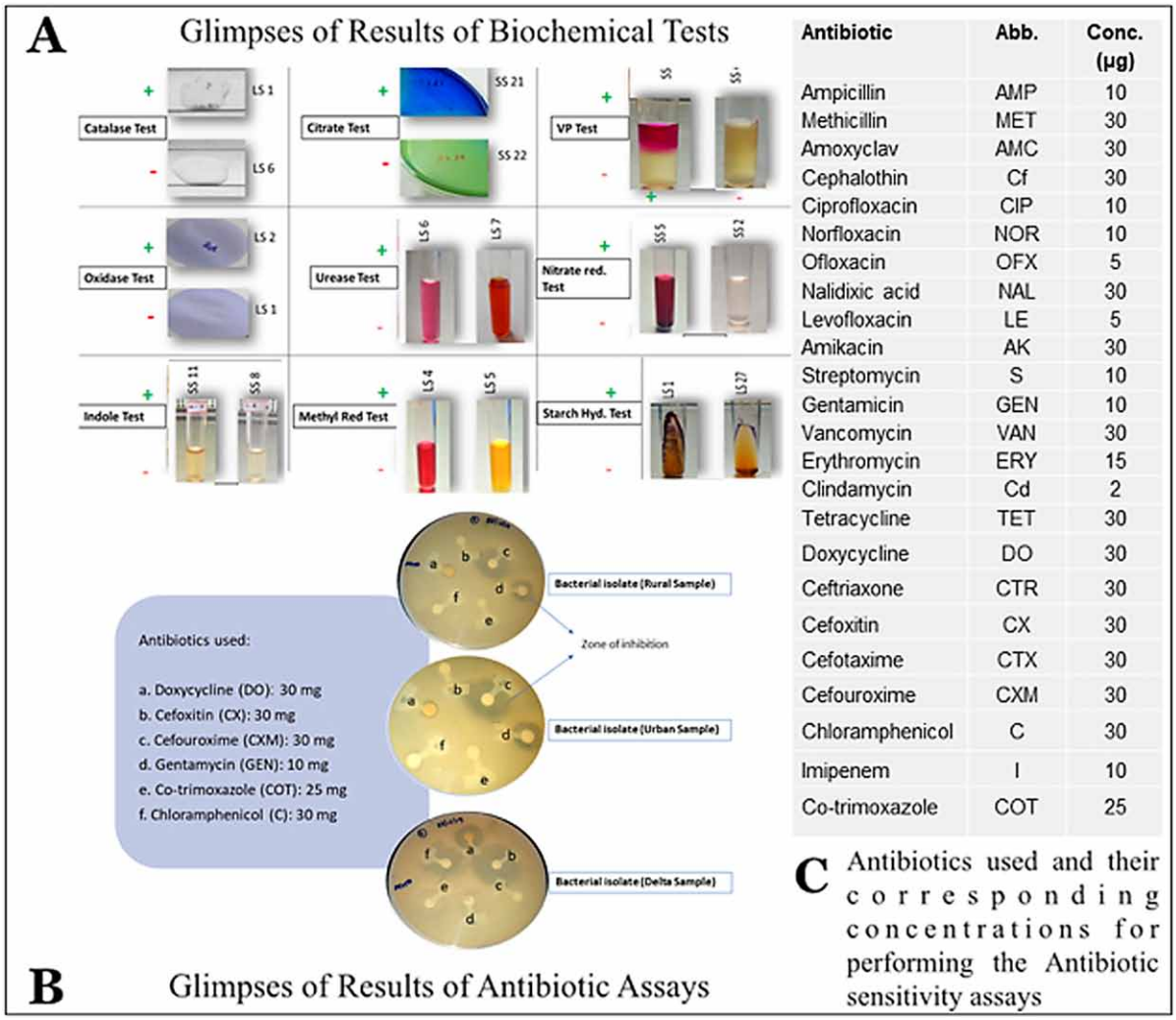
Figure 3. Predicted Disease Network from the common microbial genera across the wastewater samples



These findings led the authors to conclude that untreated medical and anthropogenic waste disposal can result in the spread of various pathogenic bacterial strains in wastewater environments of all the urban, rural and delta set ups.

Along with the computational analysis following metagenomic sequencing, this particular case study opted for a culture dependent analysis to correlate the computational data during real time lab-based experiments. Results are shown in figure 4. The figure provides the glimpses of the results of various biochemical tests (Figure 4A) followed by antibiotic sensitivity assays, the former providing initial information regarding the type of microorganism to support the Gram's staining results. These results would then be validated using PCR based amplification followed by sequencing of the 16srRNA gene from the pure culture of the individual bacterial members. In case of antibiotic sensitivity assays (Fig-

Figure 4. Glimpses of results obtained from the Biochemical and Antibiotic sensitivity assays in the wet lab-based segment of the proposed protocol. A) Representative images of results of Biochemical analysis; B) Representative images of Antibiotic sensitivity assays performed; C) List of antibiotics and their corresponding concentrations used for the analysis



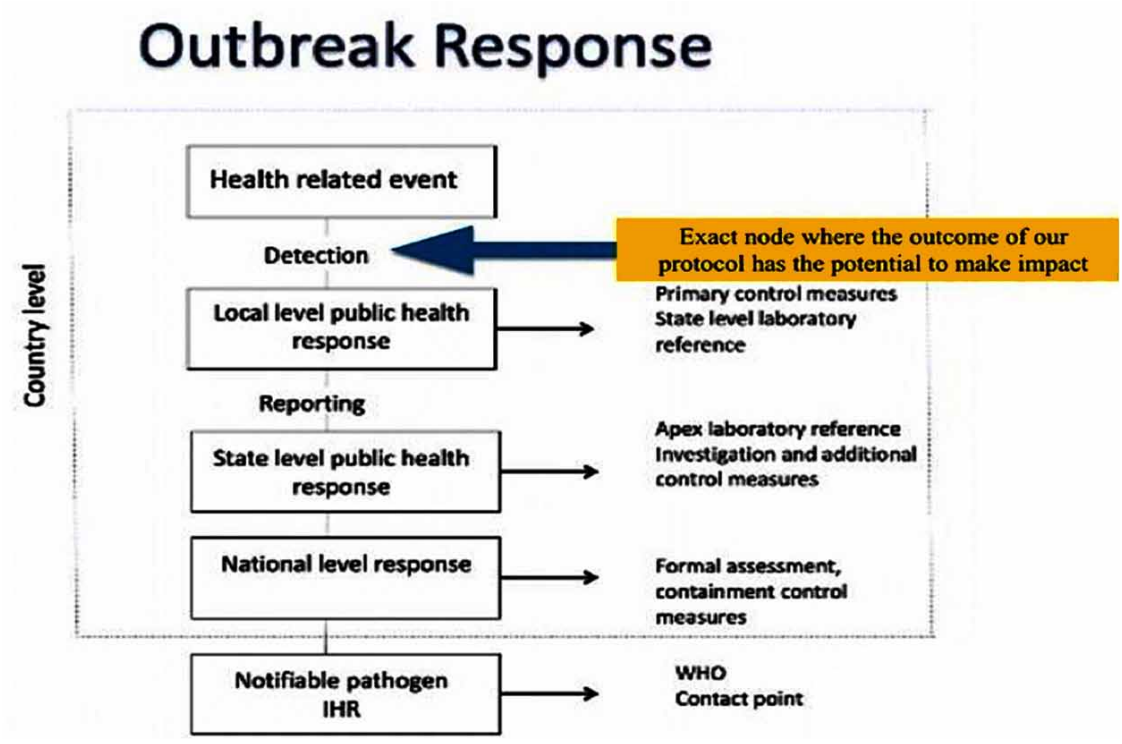
Antibiotic	Abb.	Conc. (µg)
Ampicillin	AMP	10
Methicillin	MET	30
Amoxyclav	AMC	30
Cephalothin	Cf	30
Ciprofloxacin	CIP	10
Norfloxacin	NOR	10
Ofloxacin	OFX	5
Nalidixic acid	NAL	30
Levofloxacin	LE	5
Amikacin	AK	30
Streptomycin	S	10
Gentamicin	GEN	10
Vancomycin	VAN	30
Erythromycin	ERY	15
Clindamycin	Cd	2
Tetracycline	TET	30
Doxycycline	DO	30
Ceftriaxone	CTR	30
Cefoxitin	CX	30
Cefotaxime	CTX	30
Cefouroxime	CXM	30
Chloramphenicol	C	30
Imipenem	I	10
Co-trimoxazole	COT	25

ure 4B), the presence and degree of inhibition zone (susceptibility) or the absence of inhibitory zone (resistance) enabled the authors to categorize the microorganisms into resistant and susceptible ones which were tested using various classes of antibiotics as indicated in Figure 4C. These were helpful for the authors to draw a conclusive evidence regarding the sample profiles. The culture-based method has the potential to reveal maximum levels of tolerance/minimum inhibitory concentrations, as well as the underlying antibiotic resistant mechanisms, in order to develop a comprehensive picture of antibiotic resistance in effluents and wastewaters.

APPLICATION OF THE PROTOCOL IN PUBLIC HEALTH MONITORING

The fast growth of megacities, as well as their increasing global connectivity, is becoming a major driver of new disease outbreaks. As part of risk-based surveillance, such cities should be included in early detection of anomalous illness emergence and dissemination (Nieuwenhuijse et al., 2020). The fundamentals of any outbreak teach us how to collect, document, and analyze descriptive data about people, places under specific time frames in order to generate a source hypothesis. Therefore, detection is going to be the most key facet highlighted in figure 5 as the node of interest from which the outcome of our protocol seems impactful. Local level public health response can be the primary control measures in terms of state level laboratory reference. This in turn leads to a state level public health response reporting followed by national level response. Afterwards, formal assessment as well as containment

Figure 5. Application of the protocol in outbreak



control measures can be subjected towards the application of the protocol. Finally, notifiable pathogens are subjected to international health regulations and emergency committees (IHR) in order to prevent, control, and respond to disease outbreaks around the world (Figure 5).

CONCLUSION

This research should help to understand how pathogenic and antibiotic resistant isolates propagate in wastewaters, mandating public awareness of sanitation measures. It should also allow to formulate a monitoring programme for evaluating wastewater health using a combination of culture independent and dependent molecular approaches to minimize the spread of these new pollutants in tropical environments. It can be believed that metagenomics guided environment impact assessment will be the next important topic of research in the near future, with the potential to transform policymaker's perspectives on climate change and linked healthcare, once all technical and ethical constraints are solved.

ACKNOWLEDGMENT

The authors acknowledge the contributions of Mr. Souradip Basu in the preparation of manuscript. They are also indebted to the authorities of the respective institutions for providing the necessary infrastructure for successful completion of the work.

REFERENCES

Abe, K., Nomura, N., & Suzuki, S. (2020). Biofilms: hot spots of horizontal gene transfer (HGT) in aquatic environments, with a focus on a new HGT mechanism. *FEMS Microbiology Ecology, 96*(5), fiaa031. doi:10.1093/femsec/fiaa031

Agamennone, V., Krul, C. A. M., Rijkers, G., & Kort, R. (2018). A practical guide for probiotics applied to the case of antibiotic-associated diarrhea in The Netherlands. *BMC Gastroenterology*, *18*(1), 103. doi:10.118612876-018-0831-x PMID:30078376

Aghalari, Z., Dahms, H. U., Sillanpää, M., Sosa-Hernandez, J. E., & Parra-Saldívar, R. (2020). Effectiveness of wastewater treatment systems in removing microbial agents: A systematic review. *Globalization and Health*, *16*(1), 13. doi:10.118612992-020-0546-y PMID:32013988

Ahmed, W., Angel, N., Edson, J., Bibby, K., Bivins, A., O'Brien, J. W., Choi, P. M., Kitajima, M., Simpson, S. L., Li, J., Tscharke, B., Verhagen, R., Smith, W., Zaugg, J., Dierens, L., Hugenholtz, P., Thomas, K. V., & Mueller, J. F. (2020). First confirmed detection of SARS-CoV-2 in untreated wastewater in Australia: A proof of concept for the wastewater surveillance of COVID-19 in the community. *The Science of the Total Environment*, *728*, 138764. doi:10.1016/j.scitotenv.2020.138764 PMID:32387778

Ajiboye, T. O., Oyewo, O. A., & Onwudiwe, D. C. (2021). Simultaneous removal of organics and heavy metals from industrial wastewater: A review. *Chemosphere*, *262*, 128379. doi:10.1016/j.chemosphere.2020.128379 PMID:33182079

Barcelo, D. (2020). An environmental and health perspective for COVID-19 outbreak: Meteorology and air quality influence, sewage epidemiology indicator, hospitals disinfection, drug therapies and recommendations. *Journal of Environmental Chemical Engineering*, *8*(4), 104006. doi:10.1016/j.jece.2020.104006 PMID:32373461

Bisseux, M., Colombet, J., Mirand, A., Roque-Afonso, A. M., Abravanel, F., Izopet, J., Archimbaud, C., Peigue-Lafeuille, H., Debroas, D., Bailly, J. L., & Henquell, C. (2018). Monitoring human enteric viruses in wastewater and relevance to infections encountered in the clinical setting: a one-year experiment in central France, 2014 to 2015. *Euro surveillance: bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin, 23*(7), 17-00237. doi:10.2807/1560-7917.ES.2018.23.7.17-00237

Bodor, A., Bounedjoum, N., Vincze, G. E., Erdeiné Kis, Á., Laczi, K., Bende, G., Szilágyi, Á., Kovács, T., Perei, K., & Rákhely, G. (2020). Challenges of unculturable bacteria: Environmental perspectives. *Reviews in Environmental Science and Biotechnology*, *19*(1), 1–22. doi:10.100711157-020-09522-4

Breitwieser, F. P., & Salzberg, S. L. (2020). Pavian: Interactive analysis of metagenomics data for microbiome studies and pathogen identification. *Bioinformatics (Oxford, England)*, *36*(4), 1303–1304. doi:10.1093/bioinformatics/btz715 PMID:31553437

Bürgmann, H., Frigon, D., H Gaze, W., M Manaia, C., Pruden, A., Singer, A. C., F Smets, B., & Zhang, T. (2018). Water and sanitation: an essential battlefront in the war on antimicrobial resistance. *FEMS Microbiology Ecology, 94*(9), . doi:10.1093/femsec/fiy101

Cappuccino, J. G., & Sherman, N. (2005). Microbiology: A laboratory manual. *Benjamin Cummings: New York*, *2005*, 125–179.

Chen, J., Li, J., Zhang, H., Shi, W., & Liu, Y. (2019). Bacterial Heavy-Metal and Antibiotic Resistance Genes in a Copper Tailing Dam Area in Northern China. *Frontiers in Microbiology*, *10*, 1916. doi:10.3389/fmicb.2019.01916 PMID:31481945

Chin, A., Chu, J., Perera, M., Hui, K., Yen, H. L., Chan, M., Peiris, M., & Poon, L. (2020). Stability of SARS-CoV-2 in different environmental conditions. *The Lancet Microbe*, *1*(1), e10. doi:10.1016/S2666-5247(20)30003-3 PMID:32835322

Datta, S., Rajnish, K. N., Samuel, M. S., Pugazhendhi, A., & Ethiraj, S. (2020). Metagenomic applications in microbial diversity, bioremediation, pollution monitoring, enzyme and drug discovery. A review. *Environmental Chemistry Letters*, *18*(4), 1229–1241. doi:10.100710311-020-01010-z

Daughton, C. G. (2018). Monitoring wastewater for assessing community health: Sewage Chemical-Information Mining (SCIM). *The Science of the Total Environment*, *619-620*, 748–764. doi:10.1016/j.scitotenv.2017.11.102 PMID:29161600

Deshpande, R., & Zou, C. (2020). Pseudomonas Aeruginosa Induced Cell Death in Acute Lung Injury and Acute Respiratory Distress Syndrome. *International Journal of Molecular Sciences*, *21*(15), 5356. doi:10.3390/ijms21155356 PMID:32731491

Dhariwal, A., Chong, J., Habib, S., King, I. L., Agellon, L. B., & Xia, J. (2017). MicrobiomeAnalyst: A web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Research*, *45*(W1), W180–W188. doi:10.1093/nar/gkx295 PMID:28449106

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*(5), 1792–1797. doi:10.1093/nar/gkh340 PMID:15034147

Edokpayi, J. N., Odiyo, J. O., & Durowoju, O. S. (2017). *Impact of Wastewater on Surface Water Quality in Developing Countries: A Case Study of South Africa, Water Quality, Hlanganani Tutu*. IntechOpen. Available from: https://www.intechopen.com/chapters/53194 doi:10.5772/66561

Ejtahed, H. S., Hoseini-Tavassol, Z., Khatami, S., Zangeneh, M., Behrouzi, A., Ahmadi Badi, S., Moshiri, A., Hasani-Ranjbar, S., Soroush, A. R., Vaziri, F., Fateh, A., Ghanei, M., Bouzari, S., Najar-Peerayeh, S., Siadat, S. D., & Larijani, B. (2020). Main gut bacterial composition differs between patients with type 1 and type 2 diabetes and non-diabetic adults. *Journal of Diabetes and Metabolic Disorders*, *19*(1), 265–271. doi:10.100740200-020-00502-7 PMID:32550175

European Medicines Agency and the European Centre for Disease Prevention and Control. (2009). *The bacterial challenge – time to react. A call to narrow the gap between multidrug-resistant bacteria in the EU and development of new antibacterial agents*. European Medicines Agency. Available from: https://ecdc.europa.eu/sites/portal/files/media/en/publications/Publications/0909_TER_The_Bacterial_Challenge_Time_to_React.pdf

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: A maximum likelihood approach. *Journal of Molecular Evolution*, *17*(6), 368–376. doi:10.1007/BF01734359 PMID:7288891

Felsenstein, J. (1985). Phylogenies and the Comparative Method. *American Naturalist*, *125*(1), 1–15. doi:10.1086/284325

Feng, L., Zhang, W., & Li, X. (2018). Monitoring of regional drug abuse through wastewater-based epidemiology—A critical review. *Science China. Earth Sciences*, *61*(3), 239–255. doi:10.100711430-017-9129-x

Garcias-Bonet, N., Arrieta, J. M., de Santana, C. N., Duarte, C. M., & Marbà, N. (2012). Endophytic bacterial community of a Mediterranean marine angiosperm (Posidonia oceanica). *Frontiers in Microbiology*, *3*, 342. doi:10.3389/fmicb.2012.00342 PMID:23049528

Gerhardt, P., Murray, R. G. E., Wood, W. A., & Krieg, N. R. (1994). *Methods for general and molecular bacteriology.* American Society for Microbiology. https://www.ncbi.nlm.nih.gov/

Greninger, A. L. (2018). The challenge of diagnostic metagenomics. *Expert Review of Molecular Diagnostics*, *18*(7), 605–615. doi:10.1080/14737159.2018.1487292 PMID:29898605

Guerrero-Latorre, L., Ballesteros, I., Villacrés-Granda, I., Granda, M. G., Freire-Paspuel, B., & Ríos-Touma, B. (2020). SARS-CoV-2 in river water: Implications in low sanitation countries. *The Science of the Total Environment*, *743*, 140832. doi:10.1016/j.scitotenv.2020.140832 PMID:32679506

Hassan, B., Yatoo, A. M., Khan, I. S., Ali, M. N., & Qadri, H. (2020). Determination of some commonly used antibiotic residues in hospital effluents of kashmir valley. *Uttar Pradesh Journal of Zoology*, *41*(17), 1–7. http://www.mbimph.com/index.php/UPJOZ/article/view/1703

He, Y., Yuan, Q., Mathieu, J., Stadler, L., Senehi, N., Sun, R., & Alvarez, P. J. J. (2020). Antibiotic resistance genes from livestock waste: occurrence, dissemination, and treatment. *NPJ Clean Water, 3*(4). doi:10.1038/s41545-020-0051-0

Hendriksen, R. S., Munk, P., Njage, P., van Bunnik, B., McNally, L., Lukjancenko, O., Röder, T., Nieuwenhuijse, D., Pedersen, S. K., Kjeldgaard, J., Kaas, R. S., Clausen, P., Vogt, J. K., Leekitcharoenphon, P., van de Schans, M., Zuidema, T., de Roda Husman, A. M., Rasmussen, S., Petersen, B., ... Aarestrup, F. M. (2019). Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. *Nature Communications*, *10*(1), 1124. doi:10.103841467-019-08853-3 PMID:30850636

Hendriksen, R. S., Munk, P., Njage, P., van Bunnik, B., McNally, L., Lukjancenko, O., Röder, T., Nieuwenhuijse, D., Pedersen, S. K., Kjeldgaard, J., Kaas, R. S., Clausen, P., Vogt, J. K., Leekitcharoenphon, P., van de Schans, M., Zuidema, T., de Roda Husman, A. M., Rasmussen, S., Petersen, B., ... Aarestrup, F. M. (2019). Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. *Nature Communications*, *10*(1), 1124. doi:10.103841467-019-08853-3 PMID:30850636

Holmberg, T. (2019). Animal waste work. The case of urban sewage management in Sweden. *Contemporary Social Science*, *16*(1), 1–15. doi:10.1080/21582041.2019.1630669

Hossain, M. S., Rahman, N. N., Balakrishnan, V., Puvanesuaran, V. R., Sarker, M. Z., & Kadir, M. O. (2013). Infectious risk assessment of unsafe handling practices and management of clinical solid waste. *International Journal of Environmental Research and Public Health*, *10*(2), 556–567. doi:10.3390/ijerph10020556 PMID:23435587

Karkman, A., Do, T. T., Walsh, F., & Virta, M. (2018). Antibiotic-Resistance Genes in Waste Water. *Trends in Microbiology*, *26*(3), 220–228. doi:10.1016/j.tim.2017.09.005 PMID:29033338

Khan, F. A., Söderquist, B., & Jass, J. (2019). Prevalence and Diversity of Antibiotic Resistance Genes in Swedish Aquatic Environments Impacted by Household and Hospital Wastewater. *Frontiers in Microbiology*, *10*, 688. doi:10.3389/fmicb.2019.00688 PMID:31019498

Khan, F. A., Söderquist, B., & Jass, J. (2019). Prevalence and Diversity of Antibiotic Resistance Genes in Swedish Aquatic Environments Impacted by Household and Hospital Wastewater. *Frontiers in Microbiology*, *10*, 688. doi:10.3389/fmicb.2019.00688 PMID:31019498

Kocamemi, B. A., Kurt, H., Sait, A., Sarac, F., Saatci, A. M., & Pakdemirli, B. (2020). *SARS- CoV-2 detection in Istanbul wastewater treatment plant sludges.* doi:10.1101/2020.05.12.20099358

Kolde, R. (2019). *pheatmap: Pretty Heatmaps*. R package version 1.0.12, URL https://CRAN. R-project.org/package=pheatmap

Kraemer, S. A., Ramachandran, A., & Perron, G. G. (2019). Antibiotic Pollution in the Environment: From Microbial Ecology to Public Policy. *Microorganisms*, *7*(6), 180. doi:10.3390/microorganisms7060180 PMID:31234491

Kutilova, I., Medvecky, M., Leekitcharoenphon, P., Munk, P., Masarikova, M., Davidova-Gerzova, L., Jamborova, I., Bortolaia, V., Pamp, S. J., & Dolejska, M. (2021). Extended- spectrum beta-lactamase-producing Escherichia coli and antimicrobial resistance in municipal and hospital wastewaters in Czech Republic: Culture-based and metagenomic approaches. *Environmental Research*, *193*(110487). Advance online publication. doi:10.1016/j.envres.2020.110487 PMID:33232750

La Rosa, G., Iaconelli, M., Mancini, P., Bonanno Ferraro, G., Veneri, C., Bonadonna, L., Lucentini, L., & Suffredini, E. (2020). First detection of SARS-CoV-2 in untreated wastewaters in Italy. *The Science of the Total Environment*, *736*, 139652. doi:10.1016/j.scitotenv.2020.139652 PMID:32464333

Lamastra, L., Suciu, N. A., & Trevisan, M. (2018). Sewage sludge for sustainable agriculture: Contaminants' contents and potential use as fertilizer. *Chemical and Biological Technologies in Agriculture*, *5*(10), 10. Advance online publication. doi:10.118640538-018-0122-3

Langford, B. J., So, M., Raybardhan, S., Leung, V., Soucy, J. R., Westwood, D., Daneman, N., & MacFadden, D. R. (2021). Antibiotic prescribing in patients with COVID-19: rapid review and meta-analysis. *Clinical Microbiology and Infection, 27*(4), 520–531. doi:10.1016/j.cmi.2020.12.018

Li, X., Rensing, C., Vestergaard, G., Arumugam, M., Nesme, J., Gupta, S., Brejnrod, A. D., & Sørensen, S. J. (2021). Metagenomic evidence for co-occurrence of antibiotic, biocide and metal resistance genes in pigs. *Environment International*, *158*, 106899. Advance online publication. doi:10.1016/j.envint.2021.106899 PMID:34598063

Mansfeldt, C., Deiner, K., Mächler, E., Fenner, K., Eggen, R., Stamm, C., Schönenberger, U., Walser, J. C., & Altermatt, F. (2020). Microbial community shifts in streams receiving treated wastewater effluent. *The Science of the Total Environment*, *709*, 135727. doi:10.1016/j.scitotenv.2019.135727 PMID:31887504

Mirza, A., Forbes, J. D., Zhu, F., Bernstein, C. N., Van Domselaar, G., Graham, M., Waubant, E., & Tremlett, H. (2020). The multiple sclerosis gut microbiota: A systematic review. *Multiple Sclerosis and Related Disorders*, *37*, 101427. doi:10.1016/j.msard.2019.101427 PMID:32172998

Mondal, S., & ... . (2021). A Pipeline for Assessment of Pathogenic Load in the Environment Using Microbiome Analysis. In M. Nath, D. Bhatt, P. Bhargava, & D. K. Choudhary (Eds.), *Microbial Metatranscriptomics Belowground*. Springer. doi:10.1007/978-981-15-9758-9_23

Nascimento, A. L., Souza, A. J., Andrade, P., Andreote, F. D., Coscione, A. R., Oliveira, F. C., & Regitano, J. B. (2018). Sewage Sludge Microbial Structures and Relations to Their Sources, Treatments, and Chemical Attributes. *Frontiers in Microbiology*, *9*, 1462. doi:10.3389/fmicb.2018.01462 PMID:30018612

Ngigi, A. N., Magu, M. M., & Muendo, B. M. (2019). Occurrence of antibiotics residues in hospital wastewater, wastewater treatment plant, and in surface water in Nairobi County, Kenya. *Environmental Monitoring and Assessment*, *192*(1), 18. doi:10.100710661-019-7952-8 PMID:31820117

Nieuwenhuijse, D. F., Oude Munnink, B. B., Phan, M., Hendriksen, R. S., Bego, A., Rees, C., Neilson, E. H., Coventry, K., Collignon, P., Allerberger, F., Rahube, T. O., Oliveira, G., Ivanov, I., Sopheak, T., Vuthy, Y., Yost, C. K., Tabo, D., Cuadros-Orellana, S., Ke, C., ... Koopmans, M. P. G. (2020). Setting a baseline for global urban virome surveillance in sewage. *Scientific Reports*, *10*(1), 13748. doi:10.103841598-020-69869-0 PMID:32792677

Quintela-Baluja, M., Abouelnaga, M., Romalde, J., Su, J. Q., Yu, Y., Gomez-Lopez, M., Smets, B., Zhu, Y. G., & Graham, D. W. (2019). Spatial ecology of a wastewater network defines the antibiotic resistance genes in downstream receiving waters. *Water Research*, *162*, 347–357. doi:10.1016/j.watres.2019.06.075 PMID:31295654

Rimoldi, S. G., Stefani, F., Gigantiello, A., Polesello, S., Comandatore, F., Mileto, D., Maresca, M., Longobardi, C., Mancon, A., Romeri, F., Pagani, C., Cappelli, F., Roscioli, C., Moja, L., Gismondo, M. R., & Salerno, F. (2020). Presence and infectivity of SARS-CoV-2 virus in wastewaters and rivers. *The Science of the Total Environment*, *744*, 140911. doi:10.1016/j.scitotenv.2020.140911 PMID:32693284

RStudio Team. (2020). *RStudio: Integrated Development for R.* RStudio. https://www.rstudio.com/

Sadrin, S., Sennoune, S., Gout, B., Marque, S., Moreau, J., Zinoune, K., Grillasca, J. P., Pons, O., & Maixent, J. M. (2020). A 2-strain mixture of Lactobacillus acidophilus in the treatment of irritable bowel syndrome: A placebo-controlled randomized clinical trial. *Digestive and Liver Disease, 52*(5), 534–540. doi:10.1016/j.dld.2019.12.009

Saitou, N., & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*(4), 406–425. doi:10.1093/oxfordjournals.molbev.a040454 PMID:3447015

Schneider, D., Aßmann, N., Wicke, D., Poehlein, A., & Daniel, R. (2020). Metagenomes of Wastewater at Different Treatment Stages in Central Germany. *Microbiology Resource Announcements*, *9*(15), e00201–e00220. doi:10.1128/MRA.00201-20 PMID:32273362

Serwecińska, L. (2020). Antimicrobials and Antibiotic-Resistant Bacteria: A Risk to the Environment and to Public Health. *Water (Basel)*, *12*(12), 3313. doi:10.3390/w12123313

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J. D., & Higgins, D. G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology*, *7*(1), 539. doi:10.1038/msb.2011.75 PMID:21988835

Singh, M., Ganguli, S., & Ghosh, M. M. (2019). Comparative metagenomic dataset of hospital effluent microbiome from rural and urban hospitals in West Bengal. *Data in Brief*, *25*, 104264. doi:10.1016/j.dib.2019.104264 PMID:31440549

Staley, Z. R., Chuong, J. D., Hill, S. J., Grabuski, J., Shokralla, S., Hajibabaei, M., & Edge, T. A. (2018). Fecal source tracking and eDNA profiling in an urban creek following an extreme rain event. *Scientific Reports*, *8*(1), 14390. doi:10.103841598-018-32680-z PMID:30258068

Sun, F., Xu, Z., & Fan, L. (2021). Response of heavy metal and antibiotic resistance genes and related microorganisms to different heavy metals in activated sludge. *Journal of Environmental Management*, *300*, 113754. Advance online publication. doi:10.1016/j.jenvman.2021.113754 PMID:34543965

Tedijanto, C., Olesen, S. W., Grad, Y. H., & Lipsitch, M. (2018). Estimating the proportion of bystander selection for antibiotic resistance among potentially pathogenic bacterial flora. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(51), E11988–E11995. doi:10.1073/pnas.1810840115 PMID:30559213

Thai, P. K., Ky, L. X., Binh, V. N., Nhung, P. H., Nhan, P. T., Hieu, N. Q., Dang, N., Tam, N., & Anh, N. (2018). Occurrence of antibiotic residues and antibiotic-resistant bacteria in effluents of pharmaceutical manufacturers and other sources around Hanoi, Vietnam. *The Science of the Total Environment*, *645*, 393–400. doi:10.1016/j.scitotenv.2018.07.126 PMID:30029118

The Center for Disease. (2020). *Dynamics Economics & Policy. Resistance Map: Antibiotic resistance*. https://resistancemap.cddep.org/Antibiotic Resistance. php

Thoendel, M. (2020). Targeted Metagenomics Offers Insights into Potential Tick-Borne Pathogens. *Journal of Clinical Microbiology*, *58*(11), e01893–e20. doi:10.1128/JCM.01893-20 PMID:32878948

UN WWAP (United Nations World Water Assessment Programme). (2017). *The United Nations World Water Development Report (2017). Wastewater: The Untapped Resource*. UNESCO.

Voigt, A. M., Zacharias, N., Timm, C., Wasser, F., Sib, E., Skutlarek, D., Parcina, M., Schmithausen, R. M., Schwartz, T., Hembach, N., Tiehm, A., Stange, C., Engelhart, S., Bierbaum, G., Kistemann, T., Exner, M., Faerber, H. A., & Schreiber, C. (2020). Association between antibiotic residues, antibiotic resistant bacteria and antibiotic resistance genes in anthropogenic wastewater - An evaluation of clinical influences. *Chemosphere*, *241*, 125032. doi:10.1016/j.chemosphere.2019.125032 PMID:31622887

Wang, J., Chu, L., Wojnárovits, L., & Takács, E. (2020). Occurrence and fate of antibiotics, antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARB) in municipal wastewater treatment plant: An overview. *The Science of the Total Environment*, *744*, 140997. doi:10.1016/j.scitotenv.2020.140997 PMID:32755790

Wang, X., Lan, B., Fei, H., Wang, S., & Zhu, G. (2021). Heavy metal could drive co-selection of antibiotic resistance in terrestrial subsurface soils. *Journal of Hazardous Materials*, *411*, 124848. doi:10.1016/j.jhazmat.2020.124848 PMID:33858075

Wang, Y., Pan, C. Q., & Xing, H. (2019). Advances in Gut Microbiota of Viral Hepatitis Cirrhosis. *BioMed Research International*, *9726786*, 1–9. Advance online publication. doi:10.1155/2019/9726786 PMID:31886272

WHO Media Centre. (2017). *News Release. WHO publishes list of bacteria for which new antibiotics are urgently needed*. Available from: https://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/

Wilson, D. N., Hauryliuk, V., Atkinson, G. C., & O'Neill, A. J. (2020). Target protection as a key antibiotic resistance mechanism. *Nature Reviews. Microbiology*, *18*(11), 637–648. doi:10.103841579-020-0386-z PMID:32587401

Xagoraraki, I., & O'Brien, E. (2020). Wastewater-Based Epidemiology for Early Detection of Viral Outbreaks. In Women in Water Quality (pp. 75-97). Springer. doi:10.1007/978-3-030-17819-2_5

Yang, H., Mirsepasi-Lauridsen, H. C., Struve, C., Allaire, J. M., Sivignon, A., Vogl, W., Bosman, E. S., Ma, C., Fotovati, A., Reid, G. S., Li, X., Petersen, A. M., Gouin, S. G., Barnich, N., Jacobson, K., Yu, H. B., Krogfelt, K. A., & Vallance, B. A. (2020). Ulcerative Colitis-associated E. coli pathobionts potentiate colitis in susceptible hosts. *Gut Microbes*, *12*(1), 1847976. doi:10.1080/19490976.2020.1847976 PMID:33258388

Yang, Y., Li, B., Zou, S., Fang, H. H., & Zhang, T. (2014). Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach. *Water Research*, *62*, 97–106. doi:10.1016/j.watres.2014.05.019 PMID:24937359

Yasir, M. (2020). Analysis of Microbial Communities and Pathogen Detection in Domestic Sewage Using Metagenomic Sequencing. *Diversity (Basel)*, *13*(1), 6. doi:10.3390/d13010006

Yoo, K., Yoo, H., Lee, J., Choi, E. J., & Park, J. (2020). Exploring the antibiotic resistome in activated sludge and anaerobic digestion sludge in an urban wastewater treatment plant via metagenomic analysis. *Journal of Microbiology (Seoul, Korea)*, *58*(2), 123–130. doi:10.100712275-020-9309-y PMID:31875929

Yuan, Q. B., Zhai, Y. F., Mao, B. Y., & Hu, N. (2018). Antibiotic resistance genes and intI1 prevalence in a swine wastewater treatment plant and correlation with metal resistance, bacterial community and wastewater parameters. *Ecotoxicology and Environmental Safety*, *161*, 251–259. doi:10.1016/j.ecoenv.2018.05.049 PMID:29886312

Zhang, D., Ling, H., Huang, X., Li, J., Li, W., Yi, C., Zhang, T., Jiang, Y., He, Y., Deng, S., Zhang, X., Wang, X., Liu, Y., Li, G., & Qu, J. (2020). Potential spreading risks and disinfection challenges of medical wastewater by the presence of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) viral RNA in septic tanks of Fangcang Hospital. *The Science of the Total Environment*, *741*, 140445. doi:10.1016/j.scitotenv.2020.140445 PMID:32599407

Zhong, X., Cui, P., Jiang, J., Ning, C., Liang, B., Zhou, J., Tian, L., Zhang, Y., Lei, T., Zuo, T., Ye, L., Huang, J., & Chen, H. (2021). *Streptococcus*, the Predominant Bacterium to Predict the Severity of Liver Injury in Alcoholic Liver Disease. *Frontiers in Cellular and Infection Microbiology*, *11*, 649060. doi:10.3389/fcimb.2021.649060 PMID:33816353

Zhou, Y., Wylie, K. M., El Feghaly, R. E., Mihindukulasuriya, K. A., Elward, A., Haslam, D. B., Storch, G. A., & Weinstock, G. M. (2016). Metagenomic Approach for Identification of the Pathogens Associated with Diarrhea in Stool Specimens. *Journal of Clinical Microbiology*, *54*(2), 368–375. doi:10.1128/JCM.01965-15 PMID:26637379

Zou, Q., Lin, G., Jiang, X., Liu, X., & Zeng, X. (2018, September 18). (2018). Sequence clustering in bioinformatics: An empirical study. *Briefings in Bioinformatics*. Advance online publication. doi:10.1093/bib/bby090 PMID:30239587

KEY TERMS AND DEFINITIONS

Antibiotic Resistant Bacteria (ARB): Bacteria that have changed their properties from being antibiotic-sensitive to antibiotic-resistant.

Hospital Effluent: Solids and associated wastewaters containing antibiotic residues, disinfectants, anaesthetics, organic matters, heavy metals, excreta from patients, discharged into the sewage system from clinical facilities.

Illumina Sequencing: A sequencing platform widely used to generate DNA sequences which are generated in small clusters using sequencing by synthesis approach in designated flow cells.

Metagenomics Analysis: The study of environmental samples using genetic techniques aiding microbial profiling from a mixed sample.

Pathogenic Load: A measure of the amounts of microbes in an environmental sample that can cause infections. It has a direct correlation to the severity of infectious diseases.

Outbreak Response: Set of actions such as proper hygiene, contact tracing, mapping of disease clusters, physical distancing taken in an emergency basis to cease the spread of a disease.

Wastewater Monitoring: Surveillance of municipal and clinical sludges in order to assess wastewater health, which is linked to community wellbeing.

Journal of Pharmaceutical Research International

33(53A): 274-285, 2021; Article no.JPRI.77759
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

Report of Antibiotic Resistance in Urban and Rural Wastewaters from West Bengal, India

Meesha Singh [a], Rupsha Karmakar [b], Sayak Ganguli [c*] and Mahashweta Mitra Ghosh [a]

[a] Department of Microbiology, St. Xavier's College, (Autonomous); Kolkata, India.
[b] Department of Zoology, Vivekananda College for Women, Kolkata, India.
[c] Department of Biotechnology, St. Xavier's College, (Autonomous); Kolkata, India.

Authors' contributions

This work was carried out in collaboration among all authors. Author MS performed the wet lab experiments, authors RK and SG was involved in computational analysis, authors SG and MMG conceptualised the study and evaluated the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2021/v33i53A33660
Editor(s):
(1) Dr. Ana Cláudia Coelho, University of Trás-os-Montes and Alto Douro, Portugal.
Reviewers:
(1) E. Kavitha, Sri Venkateshwara Medical College Hospital and Research Centre, India.
(2) Ahmed Sadeq Abou El-Yazed Aresha, Menoufia University, Egypt.
(3) Khaldi Amina, Université sidi bel abbes Algeria, Algeria.
Complete Peer review History, details of the editor(s), Reviewers and additional Reviewers are available here: https://www.sdiarticle5.com/review-history/77759

Original Research Article

Received 20 September 2021
Accepted 30 November 2021
Published 04 December 2021

ABSTRACT

Aims: This study aims at comparative identification of antibiotic resistance patterns in bacteria isolated from samples collected from rural environment (LS) and urban environments (SS). Metagenomic profiling gave us insights into the microbial abundance of the two samples. This study focused on culture-based methods for complete identification of antibiotic resistant isolates and estimation of comparative antibiotic resistance among the two samples.
Study Design: Untreated medical waste and anthropogenic waste disposal can lead to the propagation of different antibiotic resistant strains in wastewater environments both in urban and rural set ups which provide an insight towards this study approach mentioned in the methodology segment.
Place and Duration of Study: Sewer system of a medical facility located in Purulia, India was the collection site for liquid sludge. Solid sludge and associated wastewater were collected in vicinity of a large urban medical facility from central Kolkata, India.

**Corresponding author: E-mail: sayakgan3@gmail.com;*

Methodology: Physico-chemical properties were analyzed followed by microbiological and biochemical characterization. The antibiotic resistance patterns were determined by Kirby-Bauer disc diffusion assay. Potent multidrug resistant isolates were identified using 16srRNA gene amplification followed by Phylogenetic profiling, using CLC Genomics workbench.
Results: We observed maximum resistance in an *E. coli* isolate which was resistant up to 22 antibiotics. Combined data for resistance from urban and rural samples were found to exhibit 83.9% resistance to beta lactams, 85.7% to macrolides, 44.2% to fluoroquinolones, 50% to glycopeptides and cephalosporins, 35.7 % to carbapenems and sulfonamides, 28.5 % to tetracycline, and 23.8 % to aminoglycosides.
Conclusion: The high prevalence of antibiotic-resistant bacteria harbouring diverse resistance traits across samples indicated towards probable horizontal gene transfer across environmental niches. This study can prove to be useful to understand and map the patterns of resistance and stringently apply the counter measures related to public health practices.

Keywords: Antibiotic resistance; ARB; environmental resistome; antibiotic pollution; wastewater; Sludge.

1. INTRODUCTION

The wonder molecules which were once used as lifesaving drugs have gradually been rendered ineffective by the evolution of microorganisms which have devised mechanisms of resistance against them. Extensive use of antibiotics in healthcare and veterinary sectors along with their continuous unmonitored discharge in natural environments have successfully built up a reservoir of antibiotic resistant bacteria (ARB) which co evolve by acquisition of antibiotic resistance genes (ARG). This has led the world health organization to identify pathogenic strains of microorganisms which are potential superbugs exhibiting patterns of multidrug resistance thus directly diminishing the therapeutic potential of the antibiotics [1].

In India there is tendency of overuse of broad-spectrum penicillins owing to their availability and low cost. As a result, recent reports suggest that it is the leading country in per person antibiotic consumption which automatically results in the buildup of drug resistant pathogens [2] leading to the coinage of the phrase 'Antimicrobial resistance (AMR) capital of the world'[3].

There are several underlying factors such as inappropriate disposal of antibiotics, misuse etc. which cause intake and transfer of antibiotic resistant gene/genes in resistant bacteria [4]. The load of antibiotic resistance genes in natural environments contributes towards efficient transfer of these gene modules or collection of antibiotic resistant genes to different bacterial strains using mechanisms such as horizontal gene transfer, recombination or mutations under the selection pressure of different pollutants such as pesticides and heavy metals [5]. Some bacteria have been reported to utilize the antibiotic residues present as potential carbon source [6]. In ecological niches, the resistant genes are pooled together and as a result of this, there is an uptake of resistant genes among the bacterial neighbours horizontally. The lack of record of contamination is evident from the current reports of bacterial resistance towards third generation beta lactams and fourth generation cephalosporins [7]. There have been few reports on a lesser-known phenomenon, "environmental resistance loop" which refers to the transfer of resistant bacteria and antibiotic residues from wastewater treatment plants to the riverine systems and agricultural lands, finally reaching back to human beings and animals [8]. There is a growing concern that this phenomenon could render current and future antibiotics ineffective. According to current literatures, the mobile resistant genetic elements such as insertion sequences, transposons, integrons, and plasmids from clinical samples have been detected in hospital associated wastewaters, within a very minimal time interval following their report in hospitals [9,10]. The unique characteristics of resistome are quite evident and thus there is a dire need for an integrated approach which includes effective wastewater treatment and continuous monitoring of the resistome. The main anthropogenic sources of dissemination of ARBs are effluents from wastewater and hospital discharge where horizontal gene transfer is very dominant [11] and as a result, the "difficult to treat" infections are increasing globally [12]. The causative agents and their representative antibiotic resistance profiles are present in low percentages in wastewaters, as compared to that

in clinical settings. However, this presents a serious public health issue.

We still are in the nascent stage of our understanding regarding the types and prevalence of antibiotic resistance in the environment. Though, the standardization and organization of antibiotic resistance data of clinical origin has been curated, information regarding resistance of environmental bacteria is still very fragmented [13]. In India, according to the Resistance map resource [14] microbes resistant to aminoglycosides, carbapenems and cephalosporins pose the highest threat and have shown a steady pattern of increase from 2008 to 2020. The current pandemic has also opened a Pandora's box in terms of antibiotic usage, recent data from five different countries, suggests that there are associated bacterial infections in 6·9% of COVID-19 diagnoses of which 3·5% have been diagnosed during initial hospitalization while 14·3% post recovery. This trend is higher for patients who have received intensive critical care thus nosocomial infections cannot be ruled out [15]. This multicenter US based study also reported that almost 72% of the COVID patients were treated with antibiotics, even when there was no absolute clinical necessity [15]. The status of use of antibiotics in less developed countries are so scratchy that it is difficult to understand the actual burden that is being added to the environment as each day passes. Researchers believe that antimicrobial resistance might become even worse after COVID-19 due to their excessive use of antibiotics in humans, misuse in agriculture, and the unavailability of new formulations in the pipeline.

The aim of this study was to identify the diverse antibiotic resistant bacteria profiles in environmental wastewaters with respect to multiple antibiotics from diverse classifications. In a previous study the bacterial abundances of these two sites were reported using comparative metagenomics [16]. The metagenomic profiling gave us insights into the microbial abundance of the two samples. This study focused on culture-based methods for complete identification of antibiotic resistant isolates and estimation of comparative antibiotic resistance among the two samples.

2. MATERIALS AND METHODS

Wastewater environments were initially identified for their proximity to large medical facilities and then sample was collected as described in the previous report [16]. The microbiological culturing was initiated within 10 hours of collection. Stock samples were stored at 4°C for further processing.

2.1 Study Area

The first area of this study is Purulia. Its latitudinal and longitudinal extents are from 22°42′35′′to 23°42′00′′North and from 85°49′25′′to 86°54′37′′East. The region is climatically characterised as a dry and arid zone with parts of the Chotanagpur plateau region contributing towards its warm and humid conditions. The sample collection site was wastewater effluent collected in vicinity of a medical facility located in Purulia. The climatic conditions were recorded; Temperature: 42°C, Relative humidity: 68%, Time of collection: 12 pm.

The second area of this study, Kolkata, is located in the eastern part of India. The Calcutta Municipal Corporation has an area of 185 sq km in total. A large part of the city historically was marshy wetlands, remnants of which can still be found especially towards the eastern parts of the city [17]. The sample collection site was solid sludge and associated wastewater collected in vicinity of a large urban medical facility located in central Kolkata. The climatic conditions were recorded; Temperature: 34°C, Relative humidity: 95%, Time of collection: 11:45 am.

2.2 Evaluation of Physicochemical Parameters of the Sampling Stations

The physico-chemical parameters of the effluents were analyzed as per standardized EPA protocols [18]. The parameters analyzed were Chemical oxygen demand (COD), Total organic carbon (TOC), Nitrate, Ammonia, total Kjeldahl nitrogen, total Phosphorus and heavy metals, viz. Chromium (Cr), Mercury (Hg), Lead (Pb), Cadmium (Cd), Arsenic (As).

2.3 Microbiological Characterization

The effluent samples were serially diluted and plated onto nutrient agar plates (pH 6.6) and were incubated overnight at 37°C. Isolated colonies in higher dilution were observed on each type of plates. These colonies were then sub-cultured in nutrient agar plates for pure colony isolation and subsequently Gram stained. The colonies were observed under 100X Magnification using Bright Field Light Microscope

for morphological identification. The Gram positive and Gram-negative bacterial isolates were then presumptively identified through a series of cultural and biochemical tests according to the criteria described in Bergey's Manual of Determinative Bacteriology [19].

2.4 Biochemical Characterization

The isolates were subjected to routine biochemical tests: Catalase, Oxidase, Nitrate reduction, Indole, Methyl red, Voges-Proskauer, Citrate utilization, Urease, Starch hydrolysis, Carbohydrate fermentation [20].

2.5 Antibiotic Resistance Profiling

To determine the susceptibility of the isolates towards different antibiotics Kirby-Bauer disc diffusion assay was done. The antibiotic against which the susceptibility of the isolates was tested was selected on the basis of recommendations given in CLSI 2019, antibiotics belonging to diverse classifications and generations were used to check the sensitivity of the isolates. (Supplementary Table B). Disc diffusion assays were done using antibiotic discs from HiMedia Laboratories (Mumbai, India). Sensitivity of the isolates against antibiotics (intermediate or susceptible) was deduced on the basis of the information given in the manufacturer's manual. Results were interpreted on the basis of CLSI guidelines [21]. We classified an isolate to be multi drug resistant (MDR) if it exhibited resistance to antibiotics belonging to three or more classes.

2.6 Molecular Identification

Genomic DNA was isolated from different bacterial isolates using standard protocols and 16SrDNA fragment were amplified using universal 16S primers 27f (5′AGAGTTTGATCCTGGCTCAG3′) and 1492r (5′TACGGTTACCTTGTTACGACTT3′) [20], and sequenced. Raw sequences were assembled using the Cap3 Contig Assembly –in silico program. All the sequences were used to identify the bacteria with the help of the BLASTn program and were submitted to GenBank [22].

2.7 Phylogenetic Analysis

For constructing the phylogenetic tree, initially two programs were used to generate the multiple sequence alignment for a rapid validation of sequence scores -they were Clustal omega [23] and Muscle [24]. Based on the consensus multiple sequence alignment, phylogenetic tree was determined with 100 bootstrap replicates [25] using the phylogenetic tree module of the CLC Genomics workbench. Here both neighbour joining-based methods [26] and maximum likelihood [27] based methods were tested to evaluate the tree generated and the best consensus tree was chosen for analysis and interpretation. The tree file was visualized in Fig Tree to distinguish the different clade.

3. RESULTS AND DISCUSSION

3.1 Physico-Chemical Characterization

The physico-chemical parameters of the effluents were identified with the purpose of observing driving factors behind widespread antibiotic resistance and horizontal gene transfer. Heavy metals such as Cd, Pb, Hg were found to be higher than the permissible limits in both effluents (Fig. 1a). Heavy metals have been reported to be co-selecting agents, which promote resistance by co-resistance and cross-resistance mechanisms [28]. Total Phosphorus was higher than the permissible limits. COD was also found to be higher than the permissible limits (Fig. 1b).

3.2 Microbiological Characterization

The total number of isolated bacteria for LS and SS datasets was 19 and 23 respectively. The study was focused on multidrug resistant strains due to it was narrowed down to 14 isolates with 10 isolates from LS dataset and 4 isolates from SS dataset. Among the selected isolated bacteria, 8 were Gram negative rods (LS 2, LS 4, LS 6, LS 9, LS 17, LS 21, SS 7 and SS 19) and 6 were Gram-positive rod-shaped bacteria (LS 12, LS 14, LS 21, LS 23, LS 25 and SS 20).

3.3 Biochemical Characterization

The isolated bacteria were subjected to various biochemical tests and the results were tabulated (Supplementary Table A and C).

3.4 Antibiotic Resistance Patterns

The antibiotic resistance was determined in terms of zone of inhibition (cm) data and it was found that isolates obtained from Purulia (rural area) was found to be much higher than that in Kolkata (urban area). *E. coli* isolate (LS 2) was resistant against 22 antibiotics out of 24

antibiotics tested (Supplementary Table B) which is in conformation with the public data released by ICMR. The percentage of resistance was higher in *E. coli* isolates compared to other isolates. Some *E. coli* isolates (LS 2, LS 9, LS 17) showed variable pattern of susceptibility which may be attributed to its higher relative abundance in fecal contaminated environments or increased competence towards receiving resistant gene containing foreign plasmids [29]. A study from diarrhoea affected children in Mexico revealed 73% isolates of *E. coli* 73% resistant to ampicillin [30]. In Pakistan, [31] identified a multi antibiotic resistant isolate of *E. coli* which was resistant to cefotaxime, ceftazidime, gentamycin, ciprofloxacin, imipenem. This study also exhibits similar patterns of resistance in *E. coli.* (Table 1) (Supplementary Table D).

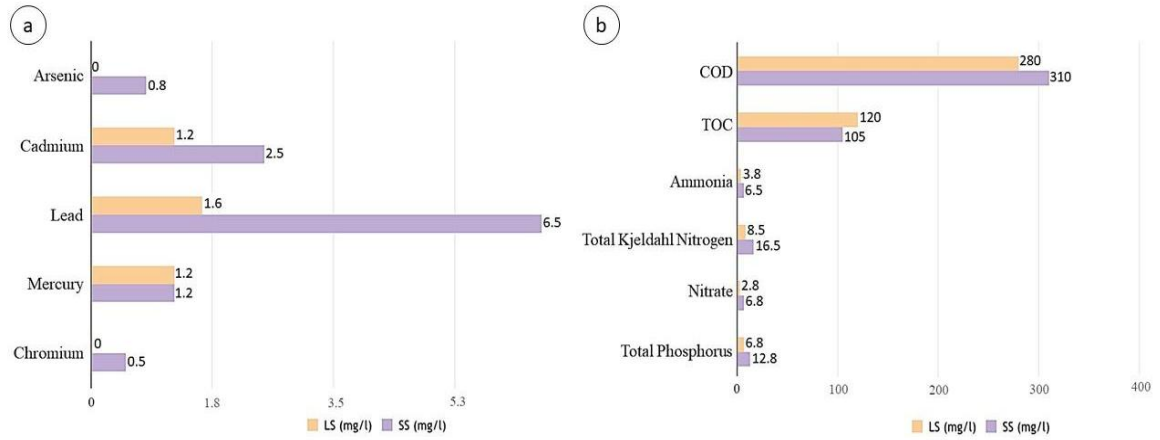


Fig. 1. Heavy metal concentrations in samples (Graphical representation of the estimation of heavy metal estimation in LS and SS sample sets with parameters on y-axis and concentrations (mg/l) on x-axis) (a) and Physico-chemical parameters of samples (Graphical representation of concentration of various analytes for LS and SS sample sets with parameters on y-axis and concentrations (mg/l) on x-axis) (b)

Table 1. Identified isolates with varying antibiotic resistance patterns

Sample ID	Isolate	Antibiotics resistant pattern
LS 2	*Escherichia coli*	AMP, CIP, GEN, NOR, CTR, NAL, AK, AMC, Cd, OFX, VAN, ERY, MET, Cf, TET, DO, CTX, IPM, CXM, LE, COT, CX
LS 4	*Shigella flexneri*	AMP, CIP, NAL, AMC, Cd, E, MET, Cf, CXM
LS 6	*Klebsiella pneumoniae*	AMP, CIP, GEN, CTR, AK, AMC, OFX, NAL, AMC, Cd, E, MET,Cf, TET, DO, CTX, IPM, CXM, COT
LS 9	*Escherichia coli*	AMP, NOR, NAL, Cd, MET, Cf
LS 12	*Bacillus safensis*	AMP, Cd, ERY, MET, Cf, CXM
LS 14	*Bacillus australimaris*	AMP, NAL, AMC, Cd, VAN, ERY, MET, Cf, CX
LS 17	*Escherichia coli*	AMP, NAL, Cd, OFX, VAN, ERY, MET
LS 21	*Comamonas aquatica*	AMP, GEN, NOR, CTR, NAL, AK, AMC, Cd, OFX, ERY, MET, Cf, TET, DO, CTX, IPM, CXM
LS 23	*Lysinibacillus fusiformis*	AMP, CIP, GEN, NOR, CTR, NAL, AK, AMC, Cd, OFX, ERY, MET, Cf, TET, CTX, IPM, CXM, COT
LS 25	*Oceanobacillus caeni*	AMP, GEN, NOR, CTR, NAL, AK, AMC, Cd, OFX, VAN, ERY, MET, Cf, CTX, IPM, CXM, COT
SS 3	*Terribacillus halophilus*	AMP, VAN, ERY, Cf
SS 7	*Citrobacter freundii*	AMP, CIP, CTR, NAL, Cd, OFX, ERY, MET, Cf, TET
SS 19	*Comamonas aquatica*	AMP, NAL, Cd, VAN, MET
SS 20	*Bacillus pumilus*	AMP, CIP, CTR, NAL, AMC, Cd, MET, Cf, CXM

K. pneumoniae is one of the commonest isolates in both hospital and community acquired infections [32]. MDR and carbapenem resistant *K. pneumoniae* has become a major therapeutic challenging scenario in several countries due to the lack of alternative existing antibiotics. Manikandan and Amsath [33] observed high degree of resistance in *K. pneumonia*e isolated from a urine sample. They reported resistance to ampicillin, ceftazidime, cefotaxime, ciprofloxacin and gentamicin. In our study, *K. pneumonia* isolate (LS 4) also exhibits resistance against beta lactams, fluoroquinolones, aminoglycoside, macrolides, tetracycline, cephalosporins, carbapenems and sulphonamide.

Several reports from around the world specially, India, Canada, Israel, Argentina, Turkey, Lebanon, Iran, China, Japan and South Korea, have reported *Shigella* spp. harbouring different types of ESBL genes [34]. In our study we found that *Shigella flexneri* isolate (LS 2) showed variable resistance towards beta lactams, fluoroquinolones, macrolides and cephalosporins.

Several strains of the commonly occurring *Lysinibacillus fusiformis* have been isolated from multiple environments including agriculture soil and factory wastewater [35]. However, there are no reports corresponding to the emergence of multidrug resistance in it. *L. fusiformis* has been reported to be sensitive to tetracycline previously [36]. In our study, *L. fusiformis* (LS 23) was found to be resistant against beta lactams, fluoroquinolones, macrolides, tetracycline, cephalosporins and sulphonamide.

Bacteria of the *Bacillus pumilus* clade are ubiquitous in a wide variety of terrestrial and marine environments, ranging from stratospheric air to deep-sea sediments and from soil to living beings [37,38, 39]. The following species belonging to *B. pumilus* clade: *B. pumilus (*LS 16), *B. safensis* (LS 21) and *B. australimaris* (LS 14) were identified in this study. The antibiotic resistance patterns of the sister clade isolates are quite similar in nature with resistance shown against beta lactams, fluoroquinolones, macrolide and cephalosporins. This presents an interesting insight into the evolutionary link of antibiotic resistance genes across various species.

Comamonas species are occasional human pathogens found in contaminated environments [40]. Their identification has been challenging with several laboratories ending up reporting them as non fermentative gram-negative bacilli that could not be further identified [41]. We have identified two *C. aquatica,* one each from both the samples (LS 17 and SS 20), which show variable resistance against beta lactams, fluoroquinolones and glycopeptide class of antibiotics. This is probably the first report of antibiotic resistance in *Comamonas* species from a waste water environment.

Oceanobacillus caeni was first isolated in South Korea as a component of activated sludge in a Bacillus-dominated wastewater treatment plant [42]. In our study the isolate (LS 22) was obtained from rural wastewater and it showed variable resistance against beta lactams, fluoroquinolones, macrolides, cephalosporins and sulphonamide. The pathology and antibiotic resistance patterns of this species have not been conclusively studied before.

Citrobacter freundii is a frequent cause of nosocomial infections and a known cause of diarrheal infections, has increasingly become multidrug resistant [43]. *C. freundii* isolate (LS 25) was identified and showed resistance against beta lactams, fluoroquinolones, aminoglycoside, macrolides, cephalosporins and tetracycline.

Terribacillus halophilus was originally isolated from field soil in Japan [44]. The isolate (SS 3) showed variable antibiotic resistance patterns against beta lactams, glycopeptides, macrolide and cephalosporin. This is the first report of occurrence of antibiotic resistance in this species. (Fig. 2a and b).

This study identifies 83.9% of the isolates to exhibit resistance to beta lactams with 100% resistance to Ampicillin [45]. Reported higher probability of occurrence of ESBL-producing *K. pneumonia* and *E. coli* isolates in hospital effluent, Waste Water Treatment Plants (WWTP) and river samples, respectively whereas, hospital effluent, sanitary effluent, outflow sewage and surface water samples were richer in quinolone resistant isolates. In this study, high macrolide resistance among the isolates (85.7%) which clearly points to the diverse antibiotic resistance potential of the effluents. The resistance to fluoroquinolones was 44.2 %, 50% for glycopeptides and cephalosporins, 35.7 % for carbapenems and sulfonamides, 28.5 % for tetracycline, 23.8% for aminoglycosides. All the isolates were however, found to be susceptible to chloramphenicol and streptomycin which may be

due to the fact that these antibiotics are prescribed very sparsely by healthcare professionals and medical practitioners in the vicinity of the collection spots.

Phylogenetic profiling revealed the presence of 4 distinct phylogenetic clusters (Table 2) (Fig. 3). The largest cluster had 5 individual OTUs (LS 4, SS 7, LS 17, LS 9, SS 19). In this group, all the isolates showed resistance against AMP, NAL, Cd, VAN and MET antibiotics. It is interesting to note that this group contains isolates obtained from different geographical and sampling conditions which indicate the possibility of genetic exchange amongst allochthonous species [46]. The cluster containing isolates LS 6, LS 2, LS 21 show resistance against a wide range of antibiotics such as beta lactams, fluoroquinolones, aminoglycoside, macrolides, tetracycline, cephalosporins, carbapenems and sulphonamides, evolving probably through Darwinian forces [47]. The sister group with OTUs obtained from different sampling conditions such as LS 14, SS 20, LS 12 show resistance against beta lactams and cephalosporins both groups inhibiting bacterial cell wall synthesis. The cluster with OTUs LS 23, SS 3 and LS 25 contains halophilic organisms with resistance against some common antibiotics such as beta lactams and cephalosporins. LS 2 and LS 6 belong to the sister clades and exhibit similar antibiotic resistance patterns viz. resistance against beta lactams, fluroquinolones, aminoglycosides, macrolides, tetracycline, cephalosporins and sulfonamide. LS 25 and SS 3 show resistance against beta lactams and glycopeptides again with the common mode of action, i.e. inhibition of cell wall synthesis. They are closely related members with respect to similarities in 16S rRNA gene sequences which justifies the observations of [48], that evolutionarily related bacteria have greater chance of being selected for in polluted environments either due to the presence of antibiotics or by the process of co selection of other pollutants (Fig. 4).

This study brings to light three important insights towards the ever-increasing burden of antimicrobial resistance in the environment, specially in wastewater. The first is the identification of multidrug resistant *E. coli* which has been included as one of the most important AMR indicators along with methicillin-resistant *Staphylococcus aureus* (MRSA) from 2019 onwards. WHO reports (https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance) suggests that 25 countries, territories and areas have provided data to the Global Antimicrobial Resistance and Use Surveillance System (GLASS) on blood-stream infections due to MRSA and 49 countries provided data on bloodstream infections due to *E. coli where* the median rate observed for methicillin-resistant S. aureus was 12.11% with an inter quartile range (IQR) of 6.4–26.4 and that for *E. coli* resistant to third generation cephalosporins was 36.0% (IQR -15.2–63.0). Thus the multidrug resistant isolate obtained from this study can also provide important insights towards the resistant gene profiles if studied further. The second important

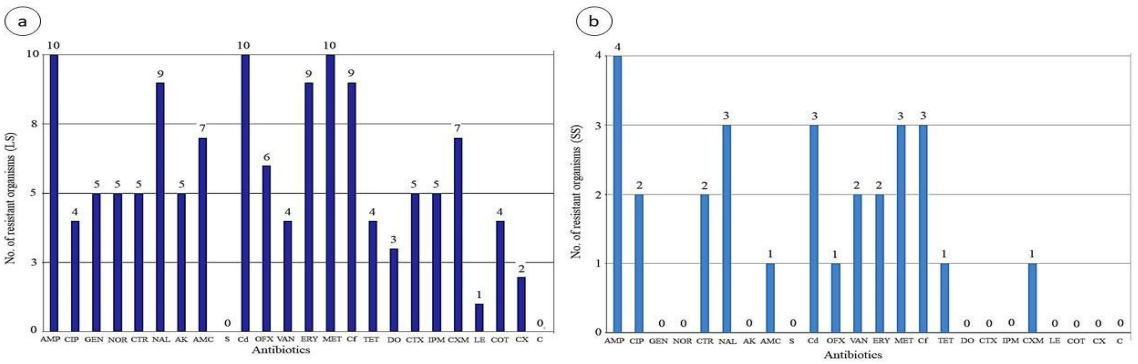


Fig. 2. Antibiotic resistant bacteria isolated from a rural setting in West Bengal, India (Graphical representation of the number of resistant isolates in LS sample set against various antibiotics; the number of resistant isolates is represented on y-axis and the antibiotics on x-axis) (a) and Antibiotic resistant bacteria isolated from an urban setting in West Bengal, India (Graphical representation of the number of resistant isolates in SS sample set against various antibiotics; the number of resistant isolates is represented on y-axis and the antibiotics on x-axis) (b)

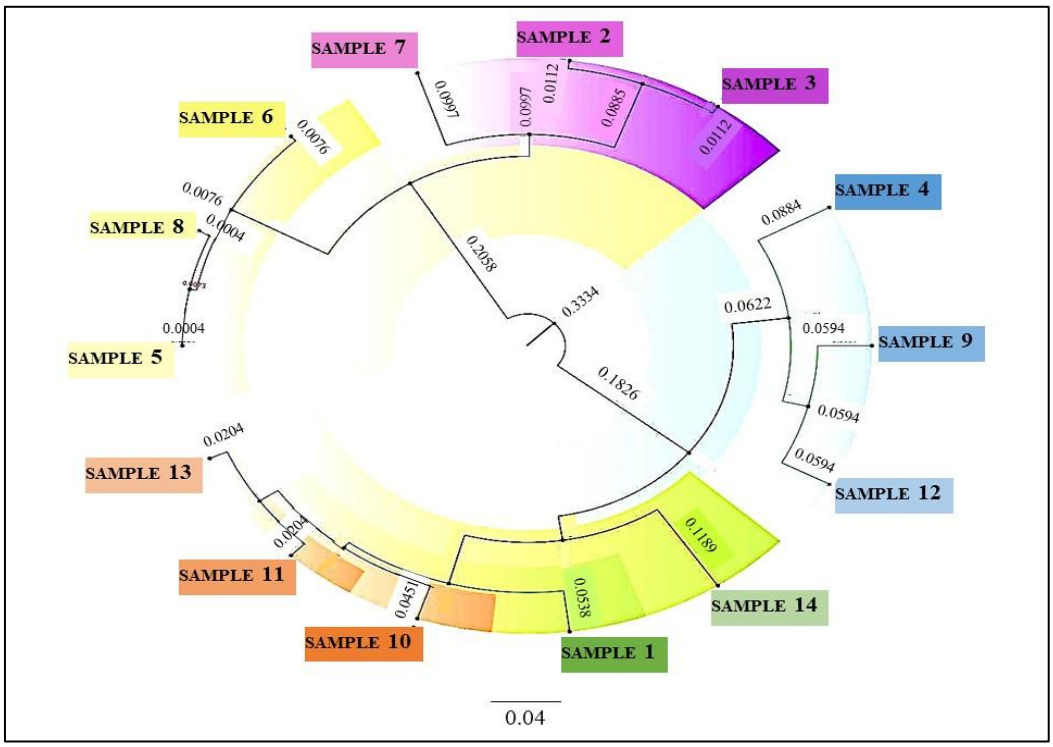


Fig. 3. Phylogenetic analysis. Four distinct clades were identified in the phylogenetic tree (Clade 1 -LS 4, SS 7, LS 17, LS 9, SS 19; Clade 2- LS 6, LS 2, LS 21; Clade 3- LS 14, SS 20, LS 12; Clade 4- LS 23, SS 3 and LS 25

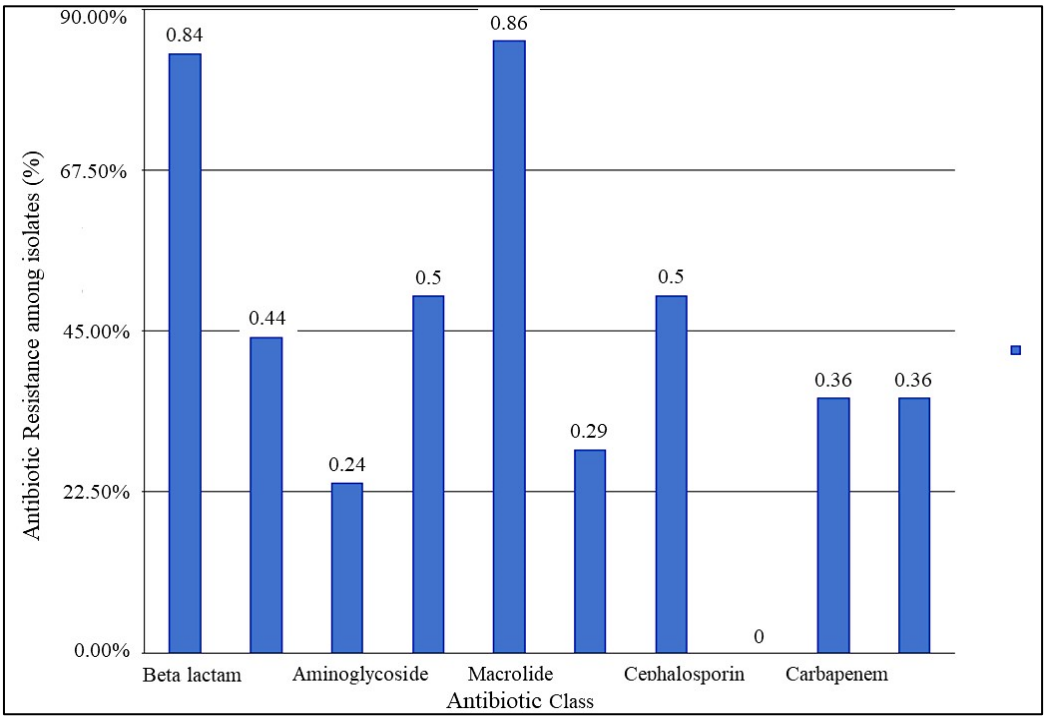


Fig. 4. Antibiotic resistance patterns of bacteria isolated from wastewaters collected from rural and urban areas of West Bengal, India. (Graphical representation of % antibiotic resistance on y-axis plotted against various classes of antibiotics on x-axis)

Table 2. Description of isolates represented in the phylogenetic tree, along with their sample IDs and GenBank Accession numbers

Phylogenetic code	Sample ID	Accession number	Bacterial identity
Sample_1	LS 4	MW380613	*Shigella flexneri*
Sample_2	LS 6	MW380614	*Klebsiella pneumoniae*
Sample_3	LS 2	MW380615	*Escherichia coli*
Sample_4	LS 23	MW380616	*Lysinibacillus fusiformis*
Sample_5	LS 14	MW380617	*Bacillus australimaris*
Sample_6	SS 20	MW380618	*Bacillus pumilus*
Sample_7	LS 21	MW380619	*Comamonas aquatica*
Sample_8	LS 12	MW380620	*Bacillus safensis*
Sample_9	SS 3	MW380621	*Terribacillus halophilus*
Sample_10	SS 7	MW380622	*Citrobacter freundii*
Sample_11	LS 17	MW380623	*Escherichia coli*
Sample_12	LS 25	MW380624	*Oceanobacillus caeni*
Sample_13	LS 9	MW380625	*Escherichia coli*
Sample_14	SS 19	MW380626	*Comamonas aquatica*

isolate exhibiting resistance is *Lysinibacillus fusiformis* which have been reported in environmental samples but with very little resistant properties. Nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) have been isolated from *Lysinibacillus fusiformis* both of which regulate the synthesis of antimicrobial compounds in the organism [49]. Thus the development of multidrug resistance in the isolate obtained in this study, probably indicates towards a mechanism in which the bacteria is able to survive competition in a particular wastewater niche as well as fortify its drug resistance mechanisms. The third interesting isolate is *Commamonas aquatica,* found in both urban and rural samples and resistant to beta lactams which has not been reported earlier. *However,* recent reports suggest the presence of antibiotic resistant *Commamonas testosteroni* in hospital set ups and in acute appendicitis [50]. It seems that the bacteria is increasing its resistance repertoire which is alarming in terms of the potential threat which it might pose in the coming years.

4. CONCLUSION

The results obtained in our analyses, leads us to believe that untreated medical waste and anthropogenic waste disposal can lead to the propagation of different antibiotic resistant strains in wastewater environments both in urban and rural set ups. It is quite evident that the isolates from rural area showed high rates of resistance as compared to that of urban area. Excreted antibiotics can end up in wastewater treatment plants which are capable of degrading the compound only partially. At the same time, such facilities probably serve as hotspots of horizontal gene transfer between bacterial species and even a few such strains if released in the environment can contribute negatively [51]. This reflects on the lack of awareness towards proper antibiotic usage in rural areas as compared to that in urban areas. This reflects on the need to practice efficient waste disposal and wastewater treatment policies. In natural settings, bacteria harboring antibiotic resistance genes are part of complex communities where they interact with other species [52].

This study should enable us to understand the route of spread of antibiotic resistant isolates in urban and rural wastewaters thus necessitating public awareness regarding measures of hygiene. Further, it should also enable us to formulate a monitoring program for evaluating wastewater health using a combination of culture independent and dependent molecular techniques to limit the spread of these emerging contaminants under tropical conditions.

SUPPLEMENTARY MATERIALS

Supplementary material is available in the followinglink:https://www.journaljpri.com/index.php/JPRI/libraryFiles/downloadPublic/22

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

The work is supported by the funds provided by the Department of Science and Technology and Biotechnology, Govt. of West Bengal, India project 236 (Sanc./ST/P&T/1G-39/2017). The authors hereby acknowledge the Department of Microbiology and Department of Biotechnology St. Xavier's College, Kolkata for providing the necessary facilities for conducting this study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Kraemer SA, Ramachandran A, Perron GG. Antibiotic pollution in the environment: From microbial ecology to public policy. Microorganisms. 2019;7(6):180. DOI: 10.3390/microorganisms7060180.
2. Klein EY, Tseng KK, Pant S, Laxminarayan R. Tracking global trends in the effectiveness of antibiotic therapy using the Drug Resistance Index. BMJ Glob Health. 2019;4(2):e001315. DOI:10.1136/bmjgh-2018-001315
3. Taneja N, Sharma M. Antimicrobial resistance in the environment: The Indian scenario. Indian J Med Res. 2019;149(2):119-128. DOI:10.4103/ijmr.IJMR_331_18
4. Manyi-Loh C, Mamphweli S, Meyer E, Okoh A. Antibiotic use in agriculture and its consequential resistance in environmental sources: Potential public health implications. Molecules. 2018;23(4):795. DOI: 10.3390/molecules23040795
5. Von Wintersdorff CJ, Penders J, van Niekerk JM, Mills ND, Majumder S, van Alphen LB, et al. Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. Front Microbiol. 2016;7:173. DOI:10.3389/fmicb.2016.00173
6. Cycoń M, Mrozik A, Piotrowska-Seget Z. Antibiotics in the Soil Environment-Degradation and Their Impact on Microbial Activity and Diversity. Front Microbiol. 2019;10:338. DOI: 10.3389/fmicb.2019.00338.
7. Chaudhry SB, Veve MP, Wagner JL. Cephalosporins: A Focus on Side Chains and β-Lactam Cross-Reactivity. Pharmacy (Basel). 2019;7(3):103. DOI: 10.3390/pharmacy7030103.
8. Cavicchioli R, Ripple WJ, Timmis KN, Azam F, Bakken LR, Baylis M, et al. Scientists' warning to humanity: microorganisms and climate change. Nat Rev Microbiol. 2019;17(9):569-586. DOI:10.1038/s41579-019-0222-5.
9. Bengtsson-Palme J, Kristiansson E, Larsson DGJ. Environmental factors influencing the development and spread of antibiotic resistance. FEMS Microbiol Rev. 2018;42(1):fux053. DOI: 10.1093/femsre/fux053.
10. Exner M, Bhattacharya S, Christiansen B, Gebel J, Goroncy-Bermes P, Hartemann P, et al. Antibiotic resistance: What is so special about multidrug-resistant Gram-negative bacteria?. GMS Hyg Infect Control. 2017;12:Doc05. DOI: 10.3205/dgkh000290.
11. Karkman A, Do TT, Walsh F, Virta MPJ. Antibiotic-Resistance Genes in Waste Water. Trends Microbiol. 2018;26(3):220-228. DOI: 10.1016/j.tim.2017.09.005.
12. Grennan D, Varughese C, Moore NM. Medications for Treating Infection. JAMA. 2020;323(1):100. DOI: 10.1001/jama.2019.17387.
13. Rizzo L, Manaia C, Merlin C, Schwartz T, Dagot C, Ploy MC, et al. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. Sci Total Environ. 2013;447:345-360. DOI: 10.1016/j.scitotenv.2013.01.032.
14. The center for disease, dynamics economics & policy. Resistance map: Antibioticresistance;2020. Available:https://resistancemap.cddep.org/ Antibiotic Resistance. php. Date accessed: January 15, 2021
15. Langford BJ, So M, Raybardhan S, Leung V, Westwood D, MacFadden DR, et al. Bacterial co-infection and secondary infection in patients with COVID-19: a living rapid review and meta-analysis. Clin Microbiol Infect. 2020;(12):1622-1629. DOI: 10.1016/j.cmi.2020.07.016.
16. Singh M, Ganguli S, Ghosh MM. Comparative metagenomic dataset of hospital effluent microbiome from rural and urban hospitals in West Bengal. Data Brief. 2019;25:104264. DOI: 10.1016/j.dib.2019.104264.
17. Available:http://kmdaonline.org/home/aar_2011

18. Lokhande RS, Singare PU, Pimple DS. Study on Physico-chemical Parameters of Waste Water Effluents from Taloja Industrial Area of Mumbai, India. Int. J. Ecosyst. 2011;1(1):1-9.
DOI:10.5923/j.ije.20110101.01
19. Bergey DH, Holt JG. Bergey's manual of determinative bacteriology. 9th ed. Baltimore: Williams & Wilkins; 1994.
20. Gerhardt P, Murray RGE, Wood WA, Krieg NR. Methods for general and molecular bacteriology. Washington D.C: American Society for Microbiology; 1994.
21. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing. 30th ed. CLSI supplement M100 (ISBN 978-1-68440-066-9 [Print]; ISBN 978-1-68440-067-6 [Electronic]). Clinical and Laboratory. Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087 USA, 2020.
22. Available: https://www.ncbi.nlm.nih.gov/
23. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Syst Biol. 2011;7:539.
DOI: 10.1038/msb.2011.75.
24. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004;32(5):1792-1797.
Available:https://doi.org/10.1093/nar/gkh340.
25. Felsenstein J. Phylogenies and the Comparative Method. Am. Nat. 1985;125(1):1-15.
26. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987;4(4):406-25.
DOI:
10.1093/oxfordjournals.molbev.a040454.
27. Felsenstein J. Evolutionary trees from DNA sequences: A maximum likelihood approach. J Mol Evol. 1981;17(6):368-376.
28. Nguyen CC, Hugie CN, Kile ML. Association between heavy metals and antibiotic-resistant human pathogens in environmental reservoirs: A review. Front Environ Sci Eng. 2019;13:46.
DOI:10.1007/s11783-019-1129-0.
29. Santos-Lopez A, Marshall CW, Scribner MR, Snyder DJ, Cooper VS. Evolutionary pathways to antibiotic resistance are dependent upon environmental structure and bacterial lifestyle. Elife. 2019;8:e47612.
DOI: 10.7554/eLife.47612.
30. Estrada-García T, Cerna JF, Paheco-Gil L, Velázquez RF, Ochoa TJ, Torres J, et al. Drug-resistant diarrheogenic Escherichia coli, Mexico. Emerg Infect Dis. 2005;11(8):1306-8.
DOI: 10.3201/eid1108.050192.
31. Sabir S, Anjum AA, Ijaz T, Ali MA, Khan MUR, Nawaz M. Isolation and antibiotic susceptibility of E. coli from urinary tract infections in a tertiary care hospital. Pak J Med Sci. 2014;30(2):389-92.
32. Caneiras C, Lito L, Melo-Cristino J, Duarte A. Community- and Hospital-Acquired *Klebsiella pneumoniae* Urinary Tract Infections in Portugal: Virulence and Antibiotic Resistance. Microorganisms. 2019;7(5):138.
DOI: 10.3390/microorganisms7050138.
33. Manikandan C, Amsath A. Antibiotic susceptibility pattern of *Klebsiella pneumoniae* isolated from urine samples. Int. J. Curr. Microbiol. App. Sci. 2013;2(8):330-337.
34. Ranjbar R, Farahani A. *Shigella*: Antibiotic-Resistance Mechanisms And New Horizons For Treatment. Infect Drug Resist. 2019;12:3137-3167.
DOI: 10.2147/IDR.S219755.
35. Ahmed I, Yokota A, Yamazoe A, Fujiwara T. Proposal of Lysinibacillus boronitolerans gen. nov. sp. nov., and transfer of Bacillus fusiformis to Lysinibacillus fusiformis comb. nov. and Bacillus sphaericus to Lysinibacillus sphaericus comb. nov. Int J Syst Evol Microbiol. 2007;57(Pt 5):1117-1125.
DOI: 10.1099/ijs.0.63867-0.
36. Priest FG, Goodfellow M, Todd C. A numerical classification of the genus Bacillus. J Gen Microbiol. 1988;134(7):1847-82.
DOI: 10.1099/00221287-134-7-1847.
PMID: 3246588.
37. Shivaji S, Chaturvedi P, Suresh K, Reddy GSN, Dutt CBS, Wainwright M, et al. Bacillus aerius sp. nov., Bacillus aerophilus sp. nov., Bacillus stratosphericus sp. nov. and Bacillus altitudinis sp. nov., isolated from cryogenic tubes used for collecting air samples from high altitudes. Int J Syst Evol Microbiol. 2006;56(Pt 7):1465-1473.
DOI: 10.1099/ijs.0.64029-0.
38. Liu Y, Lai Q, Dong C, Sun F, Wang L, Li G, et al. Phylogenetic diversity of the *Bacillus*

pumilus group and the marine ecotype revealed by multilocus sequence analysis. PLoS One. 2013;8(11):e80097.
39. Branquinho R, Klein G, Kämpfer P, Peixe LV. The status of the species Bacillus aerophilus and Bacillus stratosphericus. Request for an Opinion. Int J Syst Evol Microbiol. 2015;65(Pt 3):1101.
DOI: 10.1099/ijs.0.000004.
40. Farooq S, Farooq R, Nahvi N. *Comamonas testosteroni*: Is It Still a Rare Human Pathogen? Case Rep Gastroenterol. 2017;11(1):42-47.
DOI: 10.1159/000452197.
41. Steinberg JP, Burd EM. Other Gram-Negative and Gram-Variable Bacilli. In: Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases. 8th ed; 2015.
42. Nam JH, Bae W, Lee DH. Oceanobacillus caeni sp. nov., isolated from a Bacillus-dominated wastewater treatment system in Korea. Int J Syst Evol Microbiol. 2008;58(Pt 5):1109-1113.
DOI: 10.1099/ijs.0.65335-0.
43. Liu L, Chen D, Liu L, Lan R, Hao S, Jin W, et al. Genetic Diversity, Multidrug Resistance, and Virulence of *Citrobacter freundii* From Diarrheal Patients and Healthy Individuals. Front Cell Infect Microbiol. 2018;8:233.
DOI: 10.3389/fcimb.2018.00233.
44. An SY, Asahara M, Goto K, Kasai H, Yokota A. Terribacillus saccharophilus gen. nov., sp. nov. and Terribacillus halophilus sp. nov., spore-forming bacteria isolated from field soil in Japan. Int J Syst Evol Microbiol. 2007;57(Pt 1):51-55.
DOI: 10.1099/ijs.0.64340-0.
45. Conte D, Palmeiro JK, da Silva Nogueira K, de Lima TM, Cardoso MA, Pontarolo R, et al. Characterization of CTX-M enzymes, quinolone resistance determinants, and antimicrobial residues from hospital sewage, wastewater treatment plant, and river water. Ecotoxicol Environ Saf. 2017;136:62-69.
DOI: 10.1016/j.ecoenv.2016.10.031.
46. Lupo A, Coyne S, Berendonk TU. Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies. Front Microbiol. 2012;3:18.
DOI: 10.3389/fmicb.2012.00018.
47. Gullberg E, Cao S, Berg OG, Ilbäck C, Sandegren L, Hughes D, et al. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog. 2011;7(7): e1002158.
DOI: 10.1371/journal.ppat.1002158.
48. Martinez JL, Sanchez MB, Martinez-Solano L, Hernandez A, Garmendia L, Fajardo A, et al. Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. FEMS Microbiol Rev. 2009;33:430-449.
DOI: 10.1111/j.1574-6976.2008.00157.x.
49. Tartar SA, Tartar T. A Rare Pathogen in Acute Appendicitis: Two Cases with *Comamonas testosteroni* Infection and Literature Review. J Pediatr Infect Dis. 2020;15(02):110-112.
DOI: 10.1055/s-0038-1641604
50. Zothanpuia, Passari AK, Gupta VK, Singh BP. Detection of antibiotic-resistant bacteria endowed with antimicrobial activity from a freshwater lake and their phylogenetic affiliation. PeerJ. 2016;4:e2103.
DOI: 10.7717/peerj.2103.
51. Hiltunen T, Virta M, Laine AL. Antibiotic resistance in the wild: an eco-evolutionary perspective. Philos Trans R Soc Lond B Biol Sci. 2017;372(1712):20160039.
DOI: 10.1098/rstb.2016.0039.
52. Munita JM, Arias CA. Mechanisms of Antibiotic Resistance. Microbiol Spectr. 2016;4(2):10.1128/microbiolspec.VMBF-0016-2015.
DOI: 10.1128/microbiolspec.VMBF-0016-2015.

Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/77759