A Comparative Study on the Antimicrobial Resistance Pattern of *Klebsiella pneumoniae* Isolated from Hospital Wastewater and its Surrounding Community.

Submitted By

Ferdous Mahmod Oyishi

16336007

Anika Islam

19336012

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

Biotechnology Program,

Department of Mathematics and Natural Sciences

BRAC University

August 2023

Declaration

It is at this moment declared that:

1. The thesis submitted, titled "**A Comparative Study on the Antimicrobial Resistance Pattern of *Klebsiella pneumoniae* Isolated from Hospital Wastewater and its Surrounding Community**" is our own original work during the completion of our degree at BRAC University.
2. The thesis does not have any content that is previously published or written by a third party, except where this is appropriately cited through complete and accurate referencing.
3. The thesis does not contain any content that has been accepted or submitted for any other degree or diploma at a university or other institution.
4. We have acknowledged all primary sources of help

Student's Full Name and Signature:

Anika Islam	**Ferdous Mahmod Oyishi**
19336012	**16336007**

Approval

The Thesis Titled "**A Comparative Study on the Antimicrobial Resistance Pattern of *K. pneumoniae e* Isolated from Hospital Wastewater and its Surrounding Community**" submitted by

1. Ferdous Mahmod Oyishi

16336007

2. Anika Islam

19336012

has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Biotechnology in August 2023.

Examining Committee:

Supervisor (member)	**Akash Ahmed** **Senior Lecturer** **Department of Mathematics and Natural Science** **BRAC University**
Program coordinator (member)	**Dr. Munima Haque** **MS and BS Biotechnology Program Director & Associate Professor** **Department of Mathematics and Natural Science** **BRAC University**
Departmental Head (Chair)	**A.F.M Yusuf Haider** **Professor and Chairperson** **Department of Mathematics and Natural Sciences** **BRAC University**

Ethics Statement

For the completion of this study, we collected samples from selected venues, following all the necessary precautions. We did all the experiments at BRAC University Life Sciences Laboratory. This is to be noted that we used no animal or human models in this study.

Abstract

Klebsiella pneumoniae (Kpn) is a member of the Enterobacteriaceae which is a gram-negative, encapsulated, and non-motile bacterium. *K. pneumoniae* spreads a range of infections, including pneumonia, urinary tract infections and liver abscesses. *K. pneumoniae* is an important microorganism because it shows high resistance to carbapenems and beta- lactams. It can produce carbapenemase and extended spectrum beta-lactamase to resist this group of antibiotics. For that reason, is organism has a significant role in highly prevalent infections which are very difficult to treat.

Our study was to collect samples from three different hospitals situated in Dhaka north city corporation. Samples were collected in two categories; one sample is collected from the hospital effluents and another sample was collected from the adjacent communities of that hospitals. Then, some isolates were collected from the hospital wastewater and some isolates were collected from the community water. Then, from those isolates, *K. pneumoniae* were confirmed through PCR. Then AST was done to observe the AMR pattern of the isolates. From 177 isolates, 119 isolates were confirmed as *Klebsiella pneumoniae which is 67%* of the total isolates. In March, highest number of isolates were detected which is 30.25% of the total confirmed isolates. From Dhaka Shishu Hospital, greatest number of isolates were obtained which is 42%. Antibiotic susceptibility test was done, and the result founded that the isolates showed highest sensitivity to gentamicin (90%) and highest resistance to erythromycin (98%). Both the isolates from hospital wastewater and community water showed resistance to multiple antibiotics. Tests for AMR-genes were run and Bla_{CTXM}, Bla_{SHV}, Bla_{TEM}, Bla_{NDM} gene were found. Bla_{CTXM} was found in most of the isolates which is 40%. Bla_{SHV}, Bla_{TEM}, Bla_{NDM} these three genes were found in almost equal amount which is 20% each.

Dedication

We would like to dedicate our thesis to our parents. Thanks for always sticking with us and appreciating our efforts. Thanks for always guiding us to the fact that there is no shortcut to

success. Our parents have been relentlessly providing us with everything great that a human could be blessed with. We will be forever grateful for your relentless guidance, love and support.

This research is also dedicated to our honorable faculty members, all the thesis students of the “SPEAK” project, the lab officers, and the technicians for tenaciously helping us all through the journey.

Acknowledgment

We would like to start off by articulating our gratitude to Almighty Allah for blessing us with the opportunity and strength to accomplish this research. We are forever grateful to the Almighty for blessing us with sound health and everything good that one can ask for. Almighty Allah has kept us strong, healthy, and resolute to stick to our research work/study even amidst the global pandemic.

We acknowledge our reverence towards Professor A. F. M. Yusuf Haider, Ph.D., Professor and Chairperson of the Department of Mathematics and Natural Sciences, BRAC University, for his grants and motivation which helped us accomplish our undergraduate thesis. We are forever grateful and obliged to our respected supervisor, Akash Ahmed, Senior Lecturer, Department of Mathematics and Natural Sciences, BRAC University for his ceaseless support, supervision, persuasion, tenacious guidance, and keen motivation in generating new ideas although the research work. Additionally, our heartfelt appreciation goes towards Md Hasanuzzaman, Senior Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University for his guidance and perseverance throughout the journey.

We are ardently thankful to our Laboratory Officers Md. Mahmudul Hasan, Shamima Akhter Chowdhury, and Asma Binte Afzal for their relentless advice and cooperation. Nevertheless, we are grateful to our Research assistant, Nishat Tasnim Ananna for her assistance and support from handling laboratory equipments, designing work method to writing our report. It would have been very difficult to continue our research without their cordial intervention and enthusiasm.

Last but not the least, we would like to appreciate and thank our lab attendants Ashik-E Khuda, Tanzila Alam, and office attendant Nadira Yeasmin for their never-ending help all through the project. Lastly, we would like to appreciate and thanks our fellow lab members who assisted us during this journey and worked relentlessly alongside in the “SPEAK” Project.

Table of Contents

List of tables:

List of figures:

List of Acronyms

NI – Nosocomial Infection

ARB – Antibiotic Resistant Bacteria

WWTP – Waste-water Treatment Plans

PCR – Polymerase Chain Reaction

ARGs - Antibiotic Resistance Genes

AR - Antibiotic Resistance

DNA - Deoxyribonucleic Acid.

DNCC - Dhaka North City Corporation

NICRH - National Institute of Cancer Research & Hospital

DSH - Dhaka Shishu (Children) Hospital

AMR – Anti-microbial Resistance

TSB - Tryptic Soy Broth

MSA – Mannitol Salt Agar

NA – Nutrient Agar

LB - Luria Bertani broth

PBS - Phosphate-Buffered Saline

TE – Tris – EDTA

EDTA - Ethylenediamine Tetraacetic Acid

MCT - Micro-Centrifuge Tubes

TBE - Tris-borate-EDTA

UV – Ultra Violate

Bp – Base-pair

CLSI - Clinical and Laboratory Standards Institute

TcTS - Tube Coagulase Test

AST – Antibiotic Susceptibility Test

HAI – Healthcare Associated Infection

SA-BSI - Staphylococcus aureus Blood-stream Infection

SSSI - Skin and Skin Structure Infection

ORSA - Own Risk and Solvency Assessment

UTI – Urinary Tract Infection

RNA - Ribonucleic Acid

NI- Nosocomial infection

MDR- Multidrug-resistant

ARG- Antibiotic resistance genes

HGT- Horizontal gene transfer

HvKp- hypervirulent

ABR-Kp- antibiotic-resistant *Klebsiella pneumoniae*

MITEs- Miniature Inverted Transposable Elements

PhCs- pharmaceutical compounds

MGEs - mobile genetic elements

MIC - minimal inhibitory concentration

MCT- Micro Centrifuge Tube

RPM- Revolution per minute

Mac- MacConkey Agar

KPC- *Klebsiella pneumoniae* e carbapenemase

BPW- Buffer peptone water

AST- Antibiotic susceptibility test

Kpn- *Klebsiella pneumoniae*

ESBL- extended spectrum beta-lactamase

MHA- Muller Hilton Agar

EDTA - Ethylenediamine Tetra acetic Acid

CLSI- Clinical and Laboratory Standards Institute

CRKP--carbapenem-resistant *Klebsiella pneumoniae*

MDR- multiple-drug resistance

GLASS- Global Antimicrobial Resistance and Use Surveillance System

HCAI – Health Care Associated Infection

Chapter 1: Introduction

1.1 Background

Klebsiella pneumoniae, named after Edwin Klebs in 1875, is a Gram-negative bacterium that appears to be non-motile, encapsulated prominently by a polysaccharide capsule, rod-shaped, and non-spore-forming. It belongs to the genus Klebsiella and Enterobacteriaceae family. (Wikipedia contributors, 2023) It is also called Friedländer's bacillus, after the name of Carl Friedländer who first described it in 1882. (Klebsiella infections, 2023)

K. pneumoniae is an opportunistic pathogen that can cause both nosocomial and community-acquired pneumonia and is sometimes named Friedlander's pneumonia. (Brabb et al., 2012) *K. pneumoniae* typically inhabits in the oropharynx and gastrointestinal tract, places in the human body where it can quickly propagate the disease and cause infections upper and lower respiratory tract infection, UTI etc. (Dey et al., 2022) This is an enteric bacterium, and 5% of all healthy humans have it in their intestinal tract. People who have poor dental hygiene make a suitable habitat for *K. pneumoniae* in their oral cavity and increase their risk of getting infected by this bacterium. Alcoholics are exceptionally prone to getting infected by *K. pneumoniae*, and older adults and diabetics are also not safe from this. (Brabb et al., 2012) Moreover, life-threatening hospital-acquired infections, such as pneumonia, meningitis, bloodstream and urinary tract infections, septicemia, and soft tissue infections in enfeebled persons, can be caused by *K. pneumoniae*. (Brabb et al., 2012)

Theses hospital-acquired infections existed even before the establishment of hospitals and spreading epidemically throughout the antibiotic period. Research show that 5%–10% of the hospitalization were due to nosocomial infections in North America and Europe whereas nosocomial infection caused an above 40% of hospitalization Latin America, Sub-Saharan Africa and Asia. (Khan et al., 2015)

Epidemiological research reveals that some of the harmful nosocomial pathogens are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter baumanii*, among which *Klebsiella pneumoniae* is considered a prime one. In healthcare settings 3-7% of the nosocomial infections are caused by *K. pneumoniae*, making it the 8th crucial pathogen. (Khan et al., 2015)

The lack of proper management of hospital wastewater system and excessive use of antibiotics have taken the spreading of nosocomial infections to a more vulnerable state elevating morbidity and mortality. Like many other neighboring countries in Asia, Bangladesh doesn't abide by the proper guideline of maintaining hospital wastewater treatment plant which is creating a vulnerable pathway of spreading the nosocomial infections to the surrounding community.

Researchers found that *K. pneumoniae* have been traced in downstream water and local sediments from countries such as China, Italy, Brazil, Algeria and Europe. (Loudermilk et al., 2022) Some community-acquired infections caused by *K. pneumoniae* are liver abscesses, endophthalmitis, and meningitis, in healthy individuals. (Zhu et al., 2021)

Since the bacterium was first identified, the polysaccharide capsule has been regarded as the most distinguishing feature and most studied virulence factor. (Lawlor et al., 2005) This capsule encloses the entire cell surface, makes the organism clear on gram stain, and renders resistance against most of the host defense mechanisms. Two antigens are responsible for the pathogenicity of *K. pneumoniae* on their cell surface. The first antigen is a lipopolysaccharide (O antigen); the other is a capsular polysaccharide (K antigen). (Klebsiella infections, 2023) In vitro, the polysaccharide capsule remarkably halts the deposition of complement components onto the bacterium and profoundly diminishes the phagocytosis of the bacterium by macrophages. (Klebsiella infections, 2023) Besides, the pathogenicity of *K. pneumoniae* is also associated with the presence of some particular virulence genes that encode virulent factors, which reduce the effectiveness of the immune system. (Ahmadi et al., 2022)

On top of that, using antibiotics frequently for random viral and bacterial infections and being in contact with them has developed antibiotic-resistant *K. pneumoniae* strains, which have greatly limited the available treatment options and increased the rate of infection by Klebsiella. (Ahmadi et al., 2022)

Enzymatic and non-enzymatic mechanisms are responsible for antibiotic resistance in gram-negative bacteria. Enzymatic pathways make antibiotic-inactivating enzymes express, while gene mutations cause non-enzymatic pathways to run. (Ahmadi et al., 2022) *Klebsiella pneumoniae* is one of a few bacteria that has a high rate of antibiotic resistance owing to the organism's recurring genomic alterations. Sir Alexander Fleming was the first person to

discover the resistance of gram-negative organisms to beta-lactam antibiotics in 1929. (Ashurst & Dawson, 2023)

After that, *K. pneumoniae* was studied thoroughly and found to be producing an enzyme called beta-lactamase, which hydrolyzes the beta-lactam ring in the antibiotics, making them resistant. Researchers later discovered that *K. pneumoniae* produced extended-spectrum beta-lactamase (ESBL), which hydrolyzes oxyimino cephalosporins, making third-generation cephalosporins incompetent. This resistance against cephalosporins made carbapenems a viable treatment option for ESBL, which has a broad range of antibacterial activity against Gram-positive and Gram-negative bacteria. (Ashurst & Dawson, 2023)

However, Carbapenem-resistant Enterobacteriaceae were discovered later in 2013, among which remarkable infections were caused by *K. pneumoniae.* (Ashurst & Dawson, 2023) Carbapenem resistance is generated by the enzyme carbapenemases, and the genes that are responsible for its production are NDM, KPC, VIM, OXA, and IMP. (Joshi et al., 2023) The Carbapenem resistance leads to an up-regulation of efflux pumps, alters the outer membrane, induces the production of carbapenemase, and increases the production of ESBL enzymes in *K. pneumoniae.* (Ashurst & Dawson, 2023)

Combating *K. pneumoniae* is easier but when it becomes resistant to several antibiotics, the treatment protocol may fail. Consequently, treatment against infections by *K. pneumoniae* is getting difficult in the coming days. Multi-drug resistant (MDR) *K. pneumoniae* is one of the leading threats in this era. The UK Government's Review on Antimicrobial Resistance (AMR)anticipates nearly 10 million deaths caused by the spread of AMR by 2050. In 2019, 32 antibiotics are recognized by the WHO in clinical development that targets particular pathogens, but only six were labeled as unprecedented. (Dey et al., 2022) A European Union based study reveal that the damage caused by antibiotic resistant pathogens, specifically beta-lactamase- and carbapenemase-producing pathogens is analogous to the aggregative damage of influenza, tuberculosis, and HIV. (Loudermilk et al., 2022)

1.2 Dissemination of antibiotic-resistant bacteria (ARB) from hospital sewage to surrounding locality

As nosocomial infections (NI) are rising rapidly in a very complex manner, it is becoming increasingly difficult to manage and prevent the spread of the diseases. The increasing number of patients coming to hospitals who are susceptible to infectious diseases and the frequent occurrence of such severe infections have elevated the cost of prevention and control of NIs. The continual overuse and misuse of broad-spectrum antibiotics by these patients are adding to the worsening of the transmission of the NIs. Additionally, improper waste management in hospitals is contributing to the spread of NIs to a great extent.

Hospital emission refers to the solid, liquid, or any chemical or biological waste generated by the patients who come to receive vaccinations, diagnostic, observational, rehabilitative, and therapeutic healthcare services due to an injury or disease or due to any research activity. An exceptional quantity like 400 to 1200 L per bed of effluents is produced in hospitals every day. (Khan et al., 2015) These hospital effluents contain pathogens, harmful chemicals, heavy metals, and radioactive elements.

Hospital wastewater is firmly regarded as a reservoir for antibiotic resistance (AR) as a repercussion of the dumping of bulk antibiotic compounds, inputs of bacterial shedding, disinfectants, and metabolized drugs from patient feces, which are likely to have consisted of multidrug-resistant (MDR) pathogens. (Le et al., 2016)

Hospitals play a major role in both the emergence and spread of antimicrobial-resistant bacteria (ARB) and a generous number of these ARBs will be excreted from hospitals via wastewater systems to adjacent communities. (Hocquet et al., 2016) As a consequence, in hospital wastewater, antibiotic resistance genes (ARGs) have got a suitable place for being exchanged between clinical pathogens and other environmental bacteria in recipient sewers. This could possibly lead to broader epidemiological consequences expanding far off the hospital setting. (Le et al., 2016)

Multi-drug resistant (MDR) bacteria along with ARGs being found in the hospital wastewater making the situation out of control. The huge bulk of MDR bacteria found in hospital wastewaters could definitely enable the proliferation and dispersal of these ARGs by horizontal

gene transfer (HGT) through plasmids, transposons, and integrons. (Le et al., 2016) Integrons, being mobile genetic elements, with versatile gene acquisition systems enable horizontal gene transfer (HGT) and make an understanding of the pollution caused by the dissemination of ARGs. (An et al., 2023) Integrons are genetic materials containing a site-specific recombination system able to integrate, express and exchange specific DNA elements known as gene cassettes. (Domingues et al, 2012) The recombination and expression of mobile gene cassette arrays containing ARGs are facilitated by integrons and are being used as indicators to gather information on AR. Gram-negative bacteria precisely *K. pneumoniae* have been found to contain multiple bla genes (which encode beta-lactamase enzyme) such as blaNDM, blaKPC, blaCTX-M, and blaSHV. They are massively being released in hospital wastewaters, along with some other familiar and related drug-resistant ARGs such as qnr (fluoroquinolone), erm (macrolide), sul (sulfonamides), and tet (tetracycline) which is unquestionably a matter of concern worldwide. (Le et al., 2016)

Hospital sewage is generally released into the urban sewer system and gets mixed all together with other effluents before being treated in the sewage treatment plant which is known as co-treatment. (Al Aukidy et al., 2017) ARBs are traced in hospital sewage throughout the world but unfortunately, very few developed countries abide by the correct treatment procedure for filtering the effluents before releasing them into surface water. For illustration, the original sewage treatment method is practiced routinely in Australia, Iran, Egypt, India, Japan, South Africa, and Thailand. Nonetheless, some developing countries, like Algeria, Bangladesh, Congo, Ethiopia, India, Nepal, Pakistan, Taiwan, and Vietnam, do not follow the water treatment procedure religiously and sometimes discharge the effluents into local drainage systems, rivers, or lakes without even any previous wastewater treatment. Thus, hospital effluents can potentially become a considerable source of poisonous elements s for the aquatic environment in developing countries like ours. (Al Aukidy et al., 2017)

In order to prevent the dissemination of antimicrobial resistance by constructing strict intervention strategies, hospitals, and related institutions should have taken administering steps of surveillance on prescribing antibiotics in hospitals through a long-term evaluation process of the prescription patterns of antibiotics. (Le et al., 2016)

Past researches show that the components of wastewater namely- antimicrobials can contribute to selective pressure and elevate lateral gene transfer gaining β-lactam genes along with diverse ARGs while treating and carrying effluents. The traditional effluent treatment procedure is not developed especially to eliminate ARGs. Using disinfectant methods such as ultraviolet radiation, chlorination and ozonation universally can effectively disable pathogenic bacteria. However, reports show that these treatment procedures do not completely disable ARGs. (Loudermilk et al., 2022)

Chapter 2: Literature Review

2.1 Klebsiella pneumoniae

Klebsiella pneumoniae is a Gram-negative, non-motile, encapsulated, lactose-fermenting, facultatively anaerobic, rod-shaped bacterium. The rods are arranged singly, in pairs, or in short chains, with capsulated cells having an optimal growth temperature of 37°C. (Vanhooren et al., 1999) This non-spore-forming bacterium has a diameter of 0.3–1.0μm and a length of 0.6 - 6.0μm. (Vanhooren et al., 1999) It characteristically expresses a mucoid shape as a lactose fermenter on MacConkey agar solid medium. (Wikipedia contributors, 2023) Since the bacterium is encapsulated by a polysaccharide capsule, it gives the mucoid shape when cultured on a solid agar medium. (Brabb et al., 2012)

It is regarded as the most prominent member of the genus Klebsiella of the Enterobacteriaceae family. The genus was named Klebsiella as a tribute to the German microbiologist Edwin Klebs (1834–1913) in 1875. (Wikipedia contributors,2023) *K. pneumoniae* was first described by Carl Friedländer, a German pathologist, as an encapsulated bacteria seen to have infected immunocompromised individuals and it was confirmed after being isolated from the lungs of pneumonia patients in 1882. The species was primarily named Friedlander's bacillus, but after 1886 the bacterium preserved the name Klebsiella. (Ashurst & Dawson, 2023) *K. pneumoniae* being harmless can be commonly found in the gastrointestinal tract of mammalians but once it invades other parts of the body such as the lungs, oral cavity, and urinary tract, it can be severely harmful.

K. pneumoniae is exceptionally inhabited in the oral cavity of people with poor dental hygiene. Half of the patients who are vulnerable targets to *K. pneumoniae* and dying as a result of the infection are found to be alcoholics. Elderly people, diabetics, and immunocompromised individuals are severely susceptible to the infection of *K. pneumoniae*. According to statistics, the mortality rate of *K. pneumoniae* is greater than that of pneumococcal pneumonia where a 21% mortality rate among the general population and 64% among alcoholics. (Brabb et al., 2012)

K. pneumoniae can be grouped into three subtypes such as opportunistic (commensal), hypervirulent (hvKp), and mostly antibiotic-resistant (ABR-Kp). While ABR-KP causes most infections in patients who are immunocompromised, inducing pneumonia, bacteremia, and

urinary tract infections, in hospital settings. Hypervirulent Kp attacks healthy individuals inducing severe diseases such as liver abscesses and meningitis. Certain virulence factors of hvKp cause "metastatic" infection in multiple organs in the human body and also lead to bloodstream infections. *K. pneumoniae* is thought to be as second leading cause of bloodstream infection after E. coli. (Sydow et al., 2022)

K. pneumoniae being predominantly present in soil and water, the environment has become a pool for this very bacterium with its potentially harmful opportunistic effects. Strikingly, the environment-borne *K. pneumoniae* isolates have been found to show similar detrimental effects as with clinical isolates but differ in capsule types of the organism. Moreover, the isolates of *K. pneumoniae* found in the environment are likely to have susceptibility towards antibiotics which reveals the extreme use of broad-spectrum antibiotics in clinics. (Sydow et al., 2022)

This is why it is an infrequent cause of community-acquired pneumonia but viewed greatly as Nosocomial (hospital-acquired) pneumonia where patients are being exposed to be treated extensively with antibiotics. (Brabb et al., 2012)

Being a major source of hospital-acquired infection *K. pneumoniae* has become a prime multidrug-resistant (MDR) pathogen possessing a high morbidity and mortality rate and limited available treatment options. This species accumulates and transfers drug-resistance factors such as extended-spectrum β-lactamase (ESBL). Klebsiella has now become resistant to even carbapenem, one of the latest antibiotics. Thus, Carbapenemase-resistant *K. pneumoniae* (CRKP) is considered a threatening epidemiological concern worldwide. (Vanhooren et al., 1999)

2.2 Factors responsible for virulence of *Klebsiella pneumoniae*

The virulence of *Klebsiella pneumoniae* is controlled by diverse factors which later lead to chronic infection and antibiotic resistance in humans. *K. pneumoniae* has multifactorial

pathogenic properties such as its polysaccharide capsule, cell wall receptors, LPS, fimbriae, siderophores, adhesins, endotoxins, urease, outer-membrane proteins, and biofilms. Since its discovery in 1882, the polysaccharide capsule of *K. pneumoniae* has been considered a prime characteristic and well-studied virulence factor that has been found to halt the ejection of complement components and decrease phagocytosis of the bacterium by macrophages. The capsule has also been found to halt the proper assembly of type 1 fimbriae on its surface which later leads to the production of an extra adhesin. Consequently, isogenic capsule-negative strains have more adherent and invasive properties than wild-type strains. (Lawlor et al., 2005)

Researchers have found a capsule-negative stain get failed to colonize the bladder tissue of mice but the wild type succeeded. In the same manner, mutants without a capsule fail to possess similar lethality that a wild type would show. (Lawlor et al., 2005) Until now, researchers have studied 77 different capsular types in Klebsiella where the species that do not have a capsule tend to be less harmful. (Ashurst & Dawson, 2023)

Additionally, the cell wall receptors act as a virulent factor by helping *K. pneumoniae* to attach to host cells and change the bacterial surface with the aim of escaping phagocytosis by macrophages and polymorphonuclear leukocytes to make it easy to invade the non-phagocytic host cell. (Highsmith & Jarvis, 1985)

Furthermore, the Lipopolysaccharide (LPS), a component of the cell wall found in Gram-negative bacteria, is also considered a virulence factor having a potent immunomodulatory properties by some researchers. (Lawlor et al., 2005)

Fimbriae is another lethal factor allowing the bacterium to attach to host cells to cause infection. Additionally, siderophores are also virulent which by accumulating iron from the host cell controls the proliferation of the bacterium to infect the host cell. (Ashurst & Dawson, 2023)

Furthermore, researchers have found that an enzyme polyketide synthase (PKS) and a genotoxic metabolite named colibactin can be derived from the hypervirulent *K. pneumoniae*. *K. pneumoniae* having the pks locus facilitates gut colonization and mucosal invasion. On top of that Colibactin adds up to severe bacterial infections such as meningitis, and potentially tumorigenesis. Although *K. pneumoniae* is known to be a gut colonizer, the presence of pks and colibactin is thought to have the potential to cause colorectal cancer and probably be used

as a biomarker for determining the frequency of tumor and anticancer therapy. (Strakova et al., 2021)

In addition, a lot of adhesins derived from *K. pneumoniae* have been presumed to have virulence in host cells such as adhesin CF29K. A chromosomal region linked with allantoin metabolism may also have the potential for invasive liver infection. Moreover, there are also reports that suggest some defects in biofilm formation due to some insertional mutations have shown reduced lethality which to some extent points out the formation of biofilm to possess virulence in *K. pneumoniae*. (Lawlor et al., 2005)

Furthermore, a group study reveals that the occurrence of maximum number of infections linked with medical equipments such as urinary and intravascular catheters, resulted from the formation of biofilm by *K. pneumoniae*. They also found an exorbitant rate of 90% of these pathogenic bacteria producing biofilm to be multidrug resistant. (n.d.)

K. pneumoniae biofilm was evidently found to have grown in vitro from the late 1980s and by 1992 in vivo biofilm was discovered. Afterwards, studies done in vitro presented that 40% of *K. pneumoniae* traced from urine, blood, sputum and wound swabs were found to have produced biofilm. Along with that, 63% of *K. pneumoniae* traced from samples of urine of patients undergoing UTI treatment with catheter, were evidently found to be capable of producing biofilm in vitro. Lately, high proportion of *K. pneumoniae* strains were isolated from endotracheal tubes (ETT) in patients with ventilator-associated pneumonia (VAP), found producing in vitro biofilm pointing the fact that abiotic surfaces help them remain consistent even at higher temperature. (n.d.)

2.3 Prevalence of Carbapenem-resistant *K. pneumoniae* (Cr-KPN)

Multi-drug resistant (MDR) organisms are showing detrimental effects in the long run for the unnecessary and improper use of antimicrobial agents for decades. It has been presumed that 10 million deaths will occur worldwide within 2050 owing to the dissemination of AMR, according to the UK Government's Review on Antimicrobial Resistance. (Dey et al., 2022) In 2019, according to WHO, 32 antibiotics were recognized as working against certain pathogens in clinical development but astonishingly only six could be marked as highly novel and effective. (Dey et al., 2022)

Gram-negative bacteria acquire resistance to antibiotics in two ways such as enzymatic and non-enzymatic mechanisms. Enzymatic pathways run by expressing certain enzymes that inactivate antibiotics, whereas non-enzymatic pathways occur when gene mutations take place. (Ahmadi et al., 2022)

The pathogenesis of *K. pneumoniae* is caused by some definite virulence genes encoding virulence factors to invade host cells and cause severe infections. As time passed by the persistent use and misuse of broad-spectrum antibiotics in hospitals gave birth to innumerable antibiotic-resistant strains of each species of pathogenic bacteria. The resistant genes encoding resistant enzymes can be obtained from the bacterium itself or from Miniature Inverted Transposable Elements (MITEs) for example- the plasmid that encodes beta-lactamases or aminoglycoside-modifying enzymes. (Ahmadi et al., 2022)

K. pneumoniae is pre-eminently a pathogenic bacterium having a high rate of antibiotic resistance to the organism's continuous genomic alterations. Sir Alexander Fleming was the first person who discovered the resistance of gram-negative organisms to beta-lactam antibiotics in 1929. (Ashurst & Dawson, 2023) From there on, *K. pneumoniae* was investigated thoroughly and found to be producing an enzyme called beta-lactamase. Beta-lactamase enzyme can hydrolyze the beta-lactam ring in the antibiotics, making the producer bacterium resistant to antibiotics. Consequently, researchers found out *K. pneumoniae* to have produced extended-spectrum beta-lactamase (ESBL). (Ashurst & Dawson, 2023)

Broad-spectrum cephalosporins, monobactams, and penicillin such as class A beta-lactamases (TEM-1, TEM-2, and SHV-1), can be immobilized by Extended-Spectrum Beta-Lactamases (ESBLs). ESBLs have also been found to create resistance against ampicillin, amoxicillin, and first-generation cephalosporins. The genes got further mutated and became resistant to third-generation cephalosporins. Besides, penicillin and cephalosporins, CTX-M enzymes also include in the ESBL group showing resistance to oxyimino-beta-lactams. (Ahmadi et al., 2022)

ESBL hydrolyzing the oxyimino cephalosporins made the third-generation cephalosporins ineffective. *K. pneumoniae* being resistant against cephalosporins, made carbapenems a feasible choice for treating ESBL. Carbapenems have an extensive array of antibacterial activity against Gram-positive and Gram-negative bacteria. Nevertheless, Carbapenem-

resistant Enterobacteriaceae were found to have evolved in nature which was gradually discovered later in 2013. (Ashurst & Dawson, 2023) The group of beta-lactamases found frequently in *K. pneumoniae* isolates are *K. pneumoniae* carbapenemases (KPCs) which are wreaking towards carbapenem antibiotics. (Ahmadi et al., 2022)

On top of that, the majority of infections caused by bacteria that are carbapenem-resistant were surprisingly by *K. pneumoniae.* Carbapenem resistance generates the enzyme carbapenemases. the genes that are responsible for the production of carbapenemases are NDM, KPC, VIM, OXA, and IMP. (Joshi et al., 2023). Inside a Carbapenem-resistant bacteria, there is found to have an up-regulation of efflux pumps, alterations of the outer membrane, activation of the carbapenemase production, and an elevation of the production of ESBL enzymes in *K. pneumoniae*. (Ashurst & Dawson, 2023)

The genes that produce the beta-lactamases and carbapenemases enzymes hold significance in antibiotic resistance in *K. pneumoniae* for creating such multidrug resistance (MDR) which makes it detrimental and difficult to treat. It needs to be resistant to a minimum of one anti-bacterial drugs in three or more antimicrobial groups to certify to be MDR. (Ahmadi et al., 2022) An accessory genome of *K. pneumoniae* has been found producing a carbapenemase known as New Delhi Metallo-Beta-Lactamase 1 (NDM-1), a class B Metallo-beta-lactamase (MBL) encoded by the plasmid. Examples of some b-lactamases include carbapenemases (blaKPC and blaOXA48). (Ahmadi et al., 2022)

In the course of time, persistent use and abuse of antibiotics by clinical settings have contributed to the emergence of antibiotic-resistant *K. pneumoniae* strains and left limited treatment options against these MDRs. Without having proper knowledge and guidance on using antibiotics and the antibiotic resistance mechanisms in MDR bacteria, specifically *K. pneumoniae*, the treatment procedure will get severely difficult as well as facilitate in exacerbating the drug resistance further. (Ahmadi et al., 2022)

2.4 Prospects of Hospital wastewater in accumulating ARBs and ARGs

Nosocomial infections were severe even before the setting up of hospitals and have now become more threatening in today's antibiotic era. Researches show that 5%–10% of cases are

of nosocomial infections among all the people who are hospitalized in North America and Europe whereas in Latin America, Sub-Saharan Africa, and Asia more than 40% of people are hospitalized due to nosocomial infections. (Khan et al., 2015) Hospitals consistently require a great quantity of water each day. In a first-world country, the average requirement of water ranges from 400 to 1,200 L per bed per day but in third-world countries like ours the need fluctuates between 200 and 400L per bed per day. (Al Aukidy et al., 2017)

Hospital wastewater (HWW) is referred to as the discharge from the hospital activities like excrement from patients and hospital staff, wastes from surgery, labor, radiology, laundry, kitchen, and so on. This HWW is proven to be facilitating the spreading of ARGs and ARBs to the healthcare settings and its employees, nearby communities as well as the environment. Hospital effluents mostly contain patient excrement, surgical and hospital discards which contain loads of innumerable infective microorganisms, such as yeasts, bacteriophages, bacteria, viruses, parasites, algae, protozoa, and fungi. (Al Aukidy et al., 2017)

Hospitals use active compounds like a wide range of antibiotics on humans for various treatments. The metabolism of these active antibiotics differs widely for illustration, some of antibiotics are metabolized by 90% or more, whereas some are metabolized by only 10% or even less. The consumed antibiotics and their metabolites are excreted from patients with their urine and feces into the wastewater of hospitals. (Korzeniewska & Harnisz, 2013)

Therefore, the HWW has been considered a reservoir for virulent bacteria as well as their ARGs, hazardous pharmaceutical compounds (PhCs), heavy metals, disinfectants, and detergents. The quantity of antibiotics and antibiotic-resistant bacteria found in hospital wastewater has been found to be greater than that of municipal effluents. (Al Aukidy et al., 2017) As a consequence of the huge disposal of antibiotic compounds, disinfectants along with bacterial shedding and metabolized drugs from patient excrement, make the hospital sewage system contain an exceedingly large amount of prospective multidrug-resistant (MDR) pathogens. (Le et al., 2016)

Researchers in Asia have found Gram-negative bacteria containing numerous bla genes such as blaNDM, blaKPC, blaCTX-M, and blaSHV which are responsible for encoding the beta-lactamase enzyme in ARBs being progressively traced from hospital wastewater. Some other recurrent and associated drug-resistant ARGs like qnr (fluoroquinolone), erm (macrolide), sul

(sulfonamides), and tet (tetracycline) have also been found along with a greater quantity of MDR bacteria from hospital effluents. As a result, HWW brings forth a situation for the exchange of antibiotic resistance genes (ARGs) from clinical pathogens to the pathogens living in other recipient sewers. This transferring of ARGs possesses a wide array of epidemiological consequences far off the clinical settings only. (Le et al., 2016)

This pool of MDRs and ARBs found in HWW expedites the proliferation and spreading of ARGs by horizontal gene transfer through plasmids, transposons, and integrons. (Le et al., 2016) A study on in-depth characterization of AMR run on hospitals in Singapore quantified the presence of factors inducing antibiotic -resistance such as antibiotic residues, resistant bacteria, and genetic determinants (i.e., ARGs, integron) in hospital discharge and found to have low levels (10-fold) of ciprofloxacin, azithromycin, clarithromycin, sulfamethoxazole, and trimethoprim in municipal effluents than those of hospital effluents as these high concentration therapeutic agents are used in hospitals. (Le et al., 2016)

Furthermore, a study based on therapy for community-acquired pneumonia in Singapore where clarithromycin was used as standard medication, quantified the concentration of ARBs ranging from 105 and 106 CFU/ml differing in locations. This range of ARBs was later found in another study run on hospital wastewater in Iran. (Le et al., 2016)

Another research suggests that in Gram-negative pathogens the class 1 integrons are the reason behind the dissemination and transportation of antibiotic resistance gene cassettes. The higher the concentration of class 1 integrons in raw wastewater, the more ARB isolates were detected consistently. For instance, 97%,67%, 63%, and 76% integrons were found for E coli isolate respectively in Iran, Spain, Thailand, and the Netherlands; and 43% for Salmonella isolates in the Netherlands. This study concludes that hospital wastewater has been found to be a potential source for class 1 integrons which are directly linked to antibiotic resistance (AR). (Le et al., 2016)

These above-mentioned researches prove hospital wastewater to be a prime source of antibiotic resistance by determining resistance factors such as AB, ARB, and ARGs in tropical countries. Researchers detected higher concentrations of ARBs in hospital effluents irrespective of ward types which have the potential to cause the advent and dispersal of multi-drug resistance and

mainly antibiotic resistance if not created awareness and taken necessary steps further for proper wastewater treatment. (Le et al., 2016)

2.5 Horizontal gene transfer facilitates the transfer of ARGs

Microorganisms have the ability to have access to genetic material and can exchange genes with other microbes which results in the growth of ARGs. the gene-exchanging mechanism is accelerated through mobile genetic elements like plasmids, transposons, and bacteriophages. Researchers have determined the dissemination of plasmids carrying ARGs in clinical settings for years now. The genetic exchange in bacteria has been facilitated by Horizontal gene transfer (HGT)on the possession of antibiotic-resistant genes (ARG). Antibiotic-resistant bacteria have become a public health concern across the world. (Revista de la Academia Colombiana de Ciencias Exactas, Físicas y Naturales - Home Page, n.d.)

The low maintenance of hospital wastewater treatment, not having done any proper sewage treatment, and dumping the wastewater from the hospital directly to the municipal sewage or water bodies like rivers/lakes facilitates the spreading of multi-drug resistant bacteria. (Korzeniewska & Harnisz, 2013) The antibiotic-resistance mechanism can take place in two ways, one is by mutation of particular genes and another one is by transferring resistant genes by horizontal gene transfer (HGT). According to researchers, HGT is the most crucial factor for the present pandemic caused by AMR. (Von Wintersdorff et al., 2016)

Since the bacterial density is found to be high in raw sewage and activated sludge, the large reservoir of migratory genes can potentially transfer more from one cell to another through horizontal and vertical transfers than by mutation. These migratory genes disseminate through bacterial populations through plasmids and diverse mobile genetic elements like transposons or integrons which carry genes that encode resistance to different antibiotic elements. (Korzeniewska & Harnisz, 2013)

The horizontal gene transfer (HGT) via mobile genetic elements (MGEs) stimulates the process of disseminating ARGs and thus creates ARBs. According to WHO the spreading of pathogenic ARB has been considered as an environmental pollutant which has become a global pollution issue keeping public health in concern. (An et al., 2023)

Integrons being an MGE plays a crucial role in the propagation and transferring of ARGs globally which also provides resistance against metals and disinfectants such as qacEΔ1, and makes resistance to quaternary ammonium compounds (QACs). The integrons have the potential to conquer and manifest the exogenous genes from a huge reservoir of resistance genes and get in association with distinct mobile elements for swift transfer of ARGs among distinct species. Researchers have found that the advancement of the ARG-integron system was so accelerated by the huge dumping of improperly treated wastewater that a minimum of 1023 copies of integrons have been found to be disposed of daily into the natural environment through waste streams making ARGs an emerging environmental pollutant globally. (An et al., 2023)

The mechanism of integron is controlled by a gene named integrase (intI) along with two recombination sites. The integrons are considered ubiquitous elements traced from different environments but mostly in wastewater. Class 1 integrons have been determined as the recurrent carrier of distinct ARGs among the huge classification of integrons. In 2009, researchers found a minimum of 130 ARGs being carried by integrons which could have been potentially taken up by numerous bacterial species and residues in different environments. For illustration, dfrA1-aadA1 was discovered being transported by the integrons of Escherichia coli from hospital isolates, *K. pneumoniae* from river isolates, and Salmonella Typhimurium from food isolates, etc. (An et al., 2023)

intI mediates the construction of gene cassette array by the aggregation of gene cassettes by ARGs, following steps such as the insertion, excision, and rearrangement process. The production and transfer of the gene cassette array enable genotypic and phenotypic diversity by integrons. The integrons that carry AGRs could be collected further to the transposons and plasmids expediting their HGT into host cells. The ARG-carrying integrons could be further assembled to facilitating their HGT among host cells. (Gillings et al., 2015),

In recent times, with enormous public health concerns, studies are being conducted on integrons carrying diverse ARGs and mediating HGT for the production of MDR pathogens. (An et al., 2023)

2.5.1 Natural competence and transformation

Although *Klebsiella pneumoniae* does not show natural competence, it can take up extracellular DNA from diverse plasmids occasionally in certain circumstances. The natural competence can be escalated in *K. pneumoniae* following the incubation of cells in the presence of aminoglycoside antibiotics such as amikacin and gentamicin. (Revista de la Academia Colombiana de Ciencias Exactas, Físicas y Naturales - Home Page, n.d.)

Researchers have analyzed the transmission of genetic elements by conjugation method in a clinical isolate bearing plasmid pNDM-1 which was increased while treating with sub-lethal concentrations of antibiotics. Another research on the analysis of an RNA-seq revealed that if bacterial cells were treated with sub-lethal concentrations of amikacin, they displayed changes in the expression of several genes such as metabolic and regulatory changes. In addition, there were some variations in cell envelope components that greatly influenced the uptake of foreign DNA. The result of this research demonstrates that incorporating sub-lethal concentrations of some aminoglycosides, particularly amikacin, has the potential to foster transferring of antibiotic-resistant genes in *K. pneumoniae.* (Revista de la Academia Colombiana de Ciencias Exactas, Físicas y Naturales - Home Page, n.d.)

K. pneumoniae possesses orthologous genes that refer to the divergence of genes after a speciation event while the gene and its main function are conserved. This kind of gene can be found in bacteria that are naturally competent such as H. influenzae. However, *K. pneumoniae* does not bear the potential to take up genetic elements naturally from the environment. (Revista de la Academia Colombiana de Ciencias Exactas, Físicas y Naturales - Home Page, n.d.)
One latest research show that E. coli being phylogenetically adjacent to *K. pneumoniae*, do not regard as naturally competent, yet it was seen to have the capability to take up exogenous DNA under definite laboratory conditions.

As a consequence of this research, scientists wanted to see if the same result can be driven from *K. pneumoniae* and acquire exogenous DNA. In this regard, an amp-sensitive laboratory strain of *K. pneumoniae* (CH404) was taken following mixing with pUC19 plasmid for a free DNA source and put on a solid medium along with antibiotics. The result showed a low amount of transformants in contrast with the previous report for E. coli. After running some sequential verifying tests, it was found that although *K. pneumoniae* is not Naturally competent, it

demonstrated to be efficient enough of acquiring exogenous DNA via the transformation method, under certain laboratory conditions. (Revista de la Academia Colombiana de Ciencias Exactas, Físicas y Naturales - Home Page, n.d.)

2.5.2 Plasmid-borne ARGs are assembled by Conjugation

Antibiotic resistance (AR) in pathogenic bacteria is a ubiquitous problem requiring more exposure to its determinants so that the propagation of ARGs via HGT can be stopped immediately. Latest studies reveal that ARBs if treated with a low concentration of antibiotics facilitates resistance to antimicrobial agents, and stimulate the HGT mechanism specifically by conjugation method. Scientists established that the concentration of disinfectants such as triclosan, chlorhexidine, chlorine, and hydrogen peroxide if used below the minimal inhibitory concentration (MIC) tends to have an effect on plasmid conjugation.

Research done on antibiotics such as β-lactams and tetracycline with Sub-MIC or sub-lethal concentrations demonstrate an elevation in the conveyance of strains of S. aureus that are resistant. (Revista de la Academia Colombiana de Ciencias Exactas, Físicas y Naturales - Home Page, n.d.) According to a study published lately, antibiotics such as cefotaxime, ciprofloxacin, and ampicillin used in sublethal concentrations were found to surge the transmission of plasmids-carrying resistant genes in Escherichia coli. These researches suggest that the recurrence of conjugation transferring of plasmids carrying resistant genes has been escalated if ARBs are treated with sub-lethal concentrations of antimicrobials which can somewhat be true in the case of *K. pneumoniae.* (Revista de la Academia Colombiana de Ciencias Exactas, Físicas y Naturales - Home Page, n.d.)

2.6 Informational inadequateness observed in the current literature

Researchers have previously concentrated on the analysis of virulent/non-virulent bacteria in food, aquatic systems as well as whole environmental systems and contributed enormously to taking biological sciences to another level. In spite of this huge contribution by famous scientists and researchers, there still remain some gaps that are filled up by the burgeoning researchers following the paths of their superior ones.

The hospital wastewater (HWW) system in first-world countries is always on proper surveillance and follows all the wastewater treatment procedures religiously. In a developing

country like ours, there are mismanagements found that greatly hamper public health and need to be studied thoroughly to bring attention along with creating awareness in people.
The hospital wastewater (HWW) system in Bangladesh is yet to bring under the limelight along with the dissemination of ARGs and ARBs of pathogenic infectious bacteria. Since the HWW remains untreated and undiscovered, it plays a prime role in spreading ARGs to communities as well as different water bodies.

2.7 The Novelty of Our Study

The dissemination and propagation of antibiotic-resistant bacteria (ARB) and antibiotic-resistant genes (ARG) have become a global public health hazard. The treatment procedure and recurrence of ARGs have become alarming for the adaptation of transferring these resistant genes through horizontal gene transfer (HGT) to other bacterial species which results in a world full of multi-drug resistant (MDR) virulent bacteria. Majority of research done in Bangladesh have been focusing on the microbial threat on the food chain, and aquatic systems but never looked into the hotspot as in the hospitals where the pathogens are mostly burgeoning from and transmitted to adjacent localities.

Thus, our study focuses on the undiscovered areas of transmission of virulent *K. pneumoniae* and its ARGs from hospital wastewater to adjacent localities and hence creating public awareness as well as surveillance from Government so that proper wastewater treatment of hospital effluents is done to stop the spreading of such virulent antimicrobial resistant bacteria.

2.8 The goal, manifestation, and postulation of this study

The aim of this study is to substitute the existent research gaps and elevate our knowledge on the spreading of AMR bacteria like *K. pneumoniae* found abundantly from hospital waster and halting the health hazards associated with this opportunistic pathogen. The hypothesis of our study is the Transmission of *K. pneumoniae* from Hospital Wastewater to Adjacent Community Tap Water in Dhaka North City Corporation along with its antimicrobial resistance pattern which has been found to have detrimental consequences for Human health worldwide.

Chapter 3: Method & Materials

3.1: Sample collecting site (location)

The sample was collected from the Dhaka Metropolitan area, under the Dhaka North City Corporation. The study was conducted from February 2023 to June 2023 targeting on three important hospitals and their nearby community households. For this research, the samples were collected from each location once in every month. The selected Hospitals and their adjacent area for the research are DNCC Dedicated Covid-19 Hospital, Mohakhali, Dhaka-1212, National Institute of Cancer Research & Hospital (NICRH), and Dhaka Shishu (Children) Hospital, Shaymoli-1207. The samples were mainly collected in two categories. One is the hospital wastewater and another one is the tap water from the adjacent community houses. Taking the hospitals as the main center point, the nearby community houses or local supplied water within the range of 500m were the target for tap water/ community water. Taking into account, in our country, wastewater and dry wastages are being released into the environment without proper treatment, causing many disease outbreaks with the potential of

causing national health risks. Furthermore, the targeted areas are the places where people of every age and occupation gather. That is why, these sites were suitable for this study.

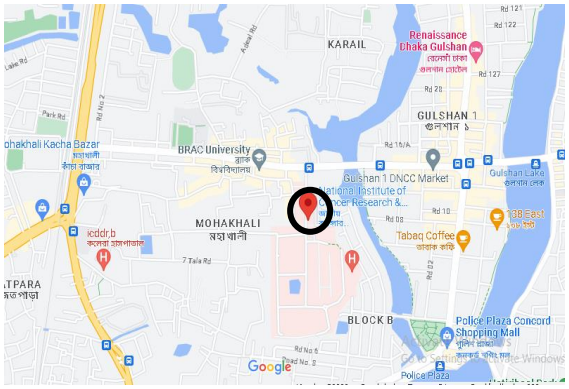


Fig3.1: location of National Cancer Hospital

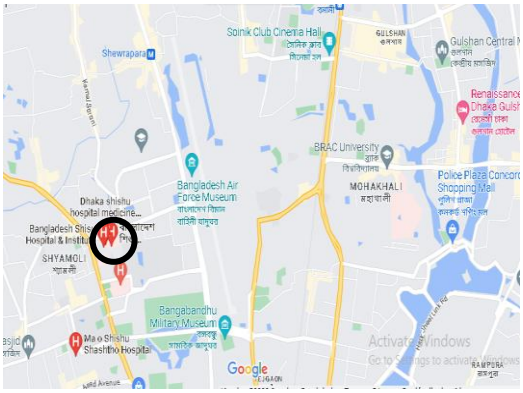


Fig 3.2: location of Dhaka Shishu Hospital

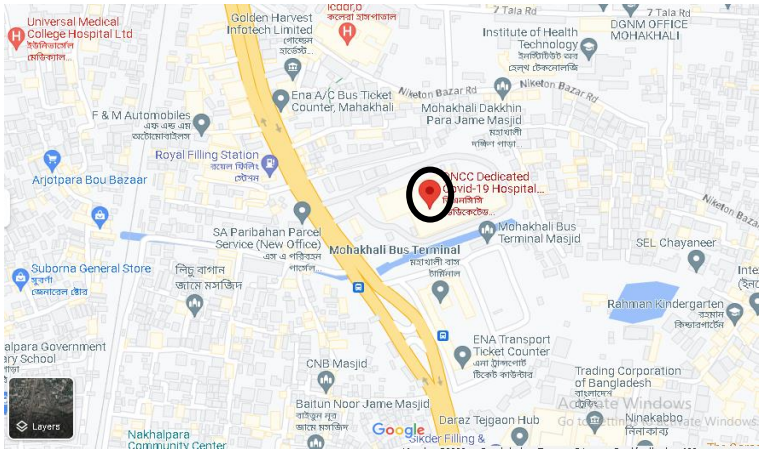


Fig3.3: Location of DNCC hospital

There is a visual representation of one of the hospitals and its dedicated community location by using google navigation:

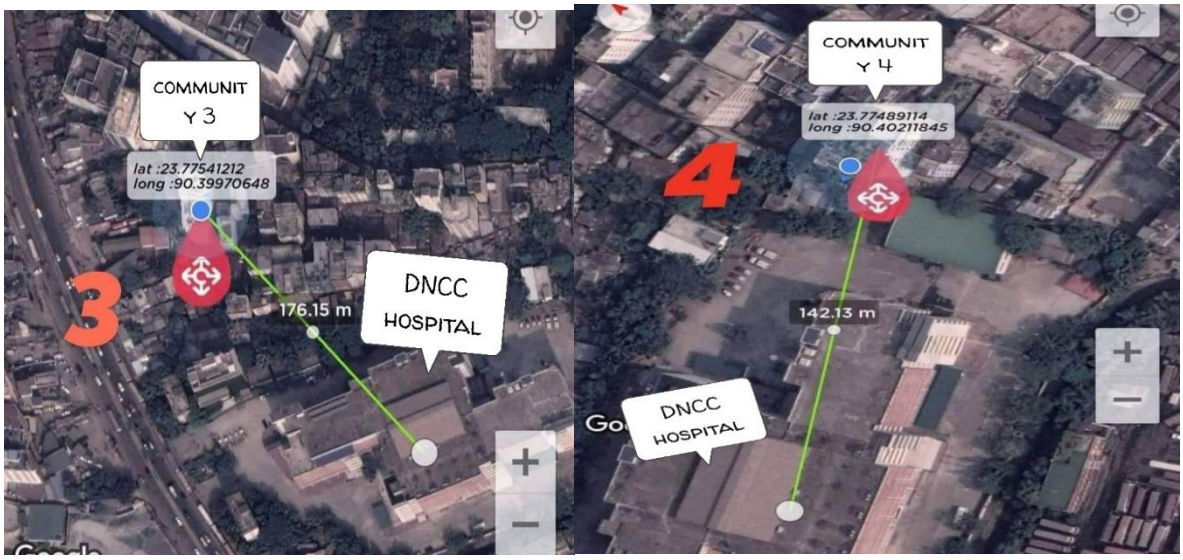


Fig 3.4: latitude and longitude of DNCC hospital and communities around it.

3.2: Sample Collecting method

Every month, samples were collected from each hospital. Hospital effluents were collected from the targeted sewerage or drain by taking proper protection. Then the community water was taken from different buildings and slum (tap and tube well), Samples were mainly collected in the early morning for the fast processing. From each hospital, one falcon full of wastewater and water from tap of four different community were collected every month. There were some precautions those were taken before collecting samples, for example, all the tools (falcon, water bottle) were autoclaved properly. To get off the cross-contamination by us and environment while collecting samples, one pair of gloves, sterilized sample collection bottles (500ml), and sterilized falcon tubes (50ml) were used. While carrying hospital wastewater and community water, a sterilized ice box and multiple ice packs were used for preserved transportation. A laboratory apron and gloves were worn by the attending parsons as a safety protocol. Wastewater samples from hospital sewerage were collected in a 50 ml sterile falcon tube. For safety, wastewater was collected twice. Tap water from the hospital's surrounding communities was collected in separated sterilized plastic bottles (500ml) and the bottles were properly labelled with unique identification names to clearly identify different community water, The falcon tubes and water collection bottles were capped properly and transferred into the icebox. After the sample collection is finished, falcons were ziplocked and the gloves thrown away. Then the hands were sanitized with 70% ethanol. Samples were then transferred to the lab for further processing within 1-2 hrs.

3.3: Steps of sample processing:

First, filtration apparatus, test tubes (10 ml), falcon tubes (50 ml), sterilized filter paper (0.45ul), modified Buffer Peptone Water or BPW (containing 5% NaCl), and normal physiological saline (0.85%NaCl), everything were autoclaved a day before the sample collection. Modified BPW was transferred into four different sterile falcon tubes under laminar airflow because there were four different community water which were to be enriched. The falcons were labelled with same unique names. From all the community tap water samples, approximately 80-100 ml of water was poured into the filtration apparatus and filtered with 0.45uL nitro-cellulose filter paper. The filter papers were then transferred with the help of sterilized pincers to the falcon tubes which contains BPW. After that, all the falcon tubes that contains samples were placed in a beaker and sealed with foil paper and masking tape, incubated in the shaker incubator at 37°C and observed for 18-24 hours. After 18-24 hours, the turbidity of BPW broth were checked and if it was satisfactory, each sample was then diluted up to 7-fold/ 8-fold in normal saline which was prepared with 0.85% NaCl. Then 100 ul from each sample 10^-2, 10^-3, 10^4 was poured on MacConkey agar media plates and spread until the liquid is dry. The media plates containing samples were then stacked together and labelled with date, group name, sample name and then kept in the incubator at 37°C for 18-24 hours. At the same time, in the day-1, wastewater from the hospital effluent was serially diluted in normal saline up to 8 folds inside the laminar. Wastewater does not need to be enriched because it us already turbid with organisms. From the diluted hospital samples, 0.1 ml of 10^-1, 10^-2, 10^-3, 10^-4, 10^-5 and 10-6 were then poured and spread evenly on MacConkey Agar media plates. The media plates were then stacked and labelled with masking tape and then placed in the incubator at 37°C for 18-24 hours until expected growth is observed.

3.4: Media, Solution and Reagents that were used

In this research, there were some media and reagents that were used in the whole process. There is the list of these reagents:

BPW: Buffer Peptone Water is a solution of peptone which is used as a pre-enrichment media for the better growth of some bacteria. It contains peptone, sodium chloride, disodium phosphate and potassium dihydrogen phosphate and the pH are 7.2(+- .2). (*BAM media M192: Buffered peptone water (BPW)*, 2020)

MacConkey Agar: MacConkey Agar is a selective and differential medium for the proper isolation of gram-negative bacteria. It is useful to isolate enteric gram-negative bacteria and differentiate lactose fermenting organism from non-fermenting organisms also. In our research, MacConkey was widely used for the spreading and streaking of *K. pneumoniae*.

KPC: *K. pneumoniae* carbapenemase media is used as a selecting media to grow Gram-negative bacilli.

MHA: Mueller-Hinton agar is not a selective media. It is a nonselective agar which is commonly used as solid growth medium in microbiology for the cultivation and antimicrobial sensitivity testing of a wide range of bacterial species. In our research, MHA media was used as the medium for antibiotic susceptibility test.

NA: Nutrient Agar medium is a multipurpose agar media with its unique composition to provide a perfect environment for the growth of a huge number of micro-organisms. It provides all the necessary nutrients needed for the growth. (Harrigan & McCance, 2966) In our research, it was used for the sub-culturing of the organism. It played a vital role to purify and distinguish the single colonies for DNA extraction and PCR.

T1N1: tryptone salt agar media tryptone or trypticase, sodium chloride, agar and distilled water. Its key ingredient is Tryptone which is the accumulation of peptides formed by the digestion of casein by protease trypsin. (ScienceDirect,2014) T1N1 was used to stock the organism for a long time in vials.

TE buffer: Tris-EDTA buffer is used in extraction of DNA.TE buffer helps to maintain the stability of DNA during extraction and storage. (Tan, 2008)

TBE Buffer: Tris-Borate-EDTA buffer is a commonly used buffer in gel electrophoresis which contains tris, boric Acid and EDTA6 for separating and analyzing nucleic acids, such as DNA and RNA. The optimal pH is 8.3. (TBE buffer (Tris-Borate-EDTA buffer) 1x, 5x & 10x, 2020)

EtBr: Ethidium Bromide is an intercalating agent used as a fluorescent dye (with UV absorbance at 300 and 360 nm) in gel electrophoresis which binds with the DNA/ RNA base pairs and helps to visualize those under UV light. (*Ethidium bromide – UK,* n.d.)

3.5 Sample processing (spread, streak) on selective media

This research was approached by the isolation and identification of *K. pneumoniae* from environmental samples, mainly from hospital wastewater and community tap water. Therefore, MacConkey Agar (HiMedia), KPC Media (selective for *K. pneumoniae* carbapenemase producing organism) (HiMedia), and Nutrient Agar (HiMedia) were used in this study. The tap water samples collected from the communities were enriched in BPW or buffer peptone water (containing 5% NaCl) broth because the turbidity of bacteria is mostly low in community tap water. KPC media is a chromogenic medium to differentiate KPC producing gram negative bacteria. It's also useful for water without selective pre-enrichment treatment. In KPC medium, *K. pneumoniae* gives circular, mucoid and greenish blue coloured morphology. In MacConkey Agar, Kpn is easily distinguished with light pink mucoid circular morphology. Then, Nutrient Agar was used for subculture and final streaking before DNA extraction and stocking.

3.6: Colony Morphology and the selection of isolates

After the incubation is done for 18-24 hours, all MacConkey media plates were brought out from the incubator, and bacterial growth and morphology were observed. The red MacConkey plates were filled with dark and light pink mucoid organisms. Every time, the total colony-forming unit/ml was counted by following the Standard plate count methods. From every sample, 5-6 light pink mucoid colonies were selected for streaking in MacConkey Agar medium again and stored in incubator at 37 degree C for 18-24 hours. In KPC media, the colonies were dark blue or greenish blue with mucoid circular colonies. The colony forming unit or CFU/mL was calculated after taking data of each spread plates by counting each single colony every time the sample was processed.

3.7: Molecular detection

3.7.1 DNA extraction

Genomic DNA isolation is a very important and common step for any molecular level experiment. For the isolation of DNA in our research, the boiling method of DNA extraction was used in this study as it is easy and less costly (Yamagishi et al., 2016). This method was used because it is so much cost effective and time saving. A pure culture from each isolate was grown in MacConkey Agar by incubating overnight at 37°C. When the desired colonies were found, a pure single colony of each isolate was taken and again straked in Nutrient Agar

medium for purer colonies for DNA extraction. It was also kept in incubator at 37°C for 18-24 hours. Then, from that streak from NA agar medium, isolates were taken into clean autoclaved MCT (micro centrifuge tube) with 150 micro litre 1x-TE buffer in the MCT.

While extracting DNA, TE buffer helps in maintaining DNA integrity. The Tris component of TE buffer serves to keep the solution's pH stable, preventing acid hydrolysis from degrading DNA. Metal ions that have the potential to cause DNA destruction are chelated by the EDTA component. (Chauhan, 2021) On the other hand, DNA pellets that may develop during the extraction and purification processes are dissolved using TE buffer. The buffer helps to solubilize the DNA and protects against its unwanted or complete denaturation or damage.
The colonies were taken by sterile loop and dissolved in TE buffer properly. Then, the MCT tubes are vortexed to mix the isolates properly. After that, the MCT tubes are sealed and labelled properly and put into the boiling machine and boiled for 15 minutes at 100-degree Celsius. Then, the tubes are removed from the machine and chilled for 5 minutes. Then, the tubes are put into the centrifuge machine and centrifuged for 5 minutes at 13,000 rpm. Then the pellets and supernatants are separated. The supernatant was collected as that was the DNA part in a freshly autoclaved MCT and sealed and labelled. Then, the DNA was stored at –20-degree Celsius. The pellets were discarded immediately. For collecting the supernatants, pipette and pipette tips were used.

3.7.2: Preparation of primer for PCR

Primers are the short fragments of DNA that helps the DNA strands to replicate. Primers are those short oligonucleotides which binds to a particular strand (opposite) of DNA and make the space for then to be amplified. There are some crucial parts of a primer which should be carefully maintained before constructing a primer, melting temperature, annealing temperature, promoter region and target sequence. There are two primers, forward and reverse primers. For the PCR process, working solution of the primers were prepared from the stock solution. Amplicon size of the primer is 133bp. The sequences of the primers are:
Forward- TGCAGATAATTCACGCCCAG (5’ - 3’)
Reverse- ACCCGCTGGACGCCAT (5’-3’)

3.7.3: Selection of controls for PCR:

To perform the PCR, a positive control was used every time. The positive control was available in the laboratory which was a true positive control of *K. pneumoniae* of laboratory standard. This positive control worked as an indicator of actual band size of *K. pneumoniae* in the gel electrophoresis. Moreover, a ladder was also used in the gel electrophoresis.

3.7.4: PCR Assay:

Polymerase Chain Reaction is a technique to amplify a specific sequence of DNA in multiple copies. PCR is helpful when numerous numbers of a particular DNA segment is needed for any kind of experiments in molecular biology, forensic analysis, evolutionary biology and medical diagnostics. (The Editors of Encyclopedia Britannica, 2023)

PCR assay of *K. pneumoniae* was conducted on PCR tubes in a completely sterile environment and inside the laminar airflow. Each PCR tubes contains 13 µl PCR mixture where 2x emerald PCR master mix was 7.5 µl, nuclease free waster was 2.5 µl and forward and reverse primers were each 0.5 µl. Generally, denaturation of the double-stranded DNA occurs between 94 and 98 degrees Celsius, annealing of the primers to the template DNA occurs between 50 and 65 degrees Celsius and primer extension by the DNA polymerase enzyme occurs at 72 degrees Celsius. Normally, these processes are performed 25 to 40 times. (De Pietro Crt, 2022) Initial denaturation of *K. pneumoniae* was at 94 degrees Celsius for 10 minutes, denaturation at 94 degrees Celsius for 30 seconds, annealing temperature is 60 degree Celsius and duration 45 seconds, elongation at 72 degrees Celsius for 45 seconds and final elongation is at 72 degrees Celsius for 10 minutes. The PCR process is run for 30 cycles.

The whole PCR process was handled with proper biosafety using all autoclaved tools and laminar. The PCR process was done in an Applied Bio-system, Thermo- Fischer. There were two control PCR tubes, positive controls and negative controls. Positive control's mixture has the DNA of confirmed *K. pneumoniae* and negative control's mixture has all the reagents accept the DNA.

3.7.5: Gel Electrophoresis:

Agarose gel electrophoresis method was used to observe the result of PCR assay and to visualize the amplificated targeted gene through bands. 6-8 µl of PCR mixture was taken from each PCR tubes and electrophoresed at 90-110 voltage for 45-60 minutes. Gel was prepared by

using 1.5% agarose and mixed with TBE buffer which contains 45 mM Tris-borate, 1 mM EDTA. (Tankeshwar, 2019) The agarose powder was properly dissolved in TBE buffer by heating process and then cooled down to approximately 55 degrees Celsius. Then 2-3 µl of ethidium bromide was added as an intercalating agent. Then, the gel was poured in the casting tray with a comb properly placed in the tray. When the gel is properly solidified, and the wells are properly created, the gel is then placed in the 1x TBE running buffer. This running buffer will help to pass the electricity. Then, the gel run is started and when its finished, the gel is observed under UV trans-illuminator and all the images are captured and archived with proper labelling. The products with the band size 133 bp or the same as the positive controls are considered as the confirmed *K. pneumoniae* Different types of ladders were used for gel electrophoresis according to the availability of the lab. Most of the time, 100 bp ladder was used for this process.

3.8: Antimicrobial susceptibility test

The isolates which were PCR confirmed went through antimicrobial susceptibility test to observe the resistance toward antibiotics. This process was done by following the Kirby-Bauer disk diffusion method along with the help of CLSI guideline. Total twelve antibiotic disks were used for AST by following the CLSI guideline. These antibiotics were: Gentamicin (10) (GEN), Amikacin (30) (AK), Cefixime (5) (CFM), Ceftriaxone (30) (CTR), Cefepime (30) (CPM), Imipenem (IPM) (10), Norfloxacin (NX) (10), Erythromycin (E) (15), Amoxiclav (Amoxicillin +clavulanic acid) (30) (AMC), Doxycycline (30) (DO), Aztreonam (30) (AT).

The isolates which were confirmed by PCR were then sub-cultured in nutrient agar or again streaked from the stock on the McConkey agar and then sub-cultured in the NA media and grown overnight at 37-degree Celsius to obtain pure single colonies of the isolates. After that, a fresh loop-full pure bacterial culture from the NA plate was properly mixed in 0.85% normal saline to make a suspension. The mixture was then compared and matched with 0.5 McFarland turbidity standard. Proper amount of suspension of the bacterial isolate was picked by a sterilized cotton swab and the lawning was done on Mueller Hinton Agar (MHA) plates. Then, by using a sterilized forceps antibiotic discs were picked carefully and placed onto the MHA agar plate by slightly pressing it to ensure complete diffusion of the discs on the medium surface. Before that, the plate was marked and divided for at least four disk on a plate. The plates were then stacked and labelled and kept in the incubator at 37C for 18- 24 hours. Then, after 18-24 hours, the zone of each disk was measured with a scale. The measurement of each

zone was taken three times and then the average measurement was noted down. Then to interpret the zone of inhibition, CLSI guideline was followed to interpret resistance, sensitivity and intermediatory zones. There is a standard scale to follow:

Table 3.8.1: Antibiotic disks interpretation measurements in (mm) guided by CLSI

Antibiotic Name	Antibiotic Class	Zone Interpretation (mm)
Gentamicin (10) (GEN)	Aminoglycosides	S>=15, I-13-14, R<=12
Amikacin (30) (AK)	Aminoglycosides	S>=17, I=15-16, R<=14
Ceftriaxone (30) (CTR)	3rd Gen cephalosporins	S>=21, I=14-20, R<=13
Cefixime (5) (CFM)	3rd Gen cephalosporins	S>=19, I=16-18, R<=15
Cefepime (30) (CPM)	4th Gen cephalosporins	S>=25, I=19-24, R<=18
Imipenem (10) (IMP)	Carbapenem	S>=16, I= 14-15, R<=13
Norfloxacin (NX) (10)	Fluoroquinolones	S>=17, I= 13-16, R<=12
Erythromycin (15) (E)	Macrolides	S>=23, I=14-22, R<=13
Amoxiclav (30) (AMC)	Amoxicillin & Clavulanic Acid	S>=18, I= 14-17, R<=13
Doxycycline (30) (DO)	Tetracyclines	S>=>14, I= 11-13, R<=<10
Aztreonam (30) (AT)	Monobactam	S>=21, I= 18-20, R<=17

3.9: Antimicrobial Resistance Gene (AMR-gene) testing:

K. pneumoniae is an opportunistic gram-negative bacterium responsible for approximately one third of all hospital and community attained infections. (Kundu et al., 2022) Infections caused by *K. pneumoniae* are being difficult to control because of the worldwide emergence of carbapenem-resistant isolates as many of the isolates produce carbapenemase due to the presence of some specific gene. (Awoke et al., 2022) Carbapenems are beta lactam antibiotics with beta lactam ring that are more stable against most of the beta lactamases. These are very effective against gram-negative bacteria. Many *K. pneumoniae* have become carbapenem and ESBL resistant in environment. So, it was another important target of our research to find out

if there is any carbapenem resistance or ESBL resistance pattern in the AST process and then finding out the responsible genes through further gene specific PCR based identification.

In order to detect the genes that are responsible for the resistance of carbapenems and beta-lactams, eight genes were targeted which are *bla*$_{NDM}$, *bla*$_{KPC}$, and *bla*$_{OXA48}$, *bla*$_{VIM,}$ *bla*$_{CTXM-15}$, *bla*$_{SHV}$, *bla*$_{TEM}$, and *bla*$_{IMP.}$ (Kundu et al., 2022) *bla*$_{NDM}$, *bla*$_{KPC}$, *bla*$_{OXA48}$, *bla*$_{VIM}$ and *bla*$_{IMP}$ are the gene responsible for carbapenemase production. *Bla*$_{CTXM,}$ *bla*$_{SHV}$ and *bla*$_{TEM}$ are responsible for ESBL production. (Awoke et al., 2022) For the presence of these genes, some bacteria become resistant to antibiotics and the disease is then hard to treat. Here are the primers used for these PCR:

Table 3.9.1: primer sequences for all the AMR-genes used in this research (Dallenne et al., 2010)

Primer name:	**Primer sequence:**	**Amplicon size:**	**Reference:**
bla TEM	F-AAAATTCTTGAAGACG, R- TTACCAATGCTTAATCA	1100-1200	Dallenne et al., 2010
bla CTX-M	F-ACGCTGTTGTTAGGAAGTG, R-TTGAGGCTGGGTGAAGT	759	Dallenne et al., 2010
bla-SHV	F-TACCATGAGCGATAACAGCG R-GATTTGCTGATTTCGCTCGG	450	Dallenne et al., 2010
NDM-1	F-GGTTTGGCGATCTGGTTTTC R-CGGAATGGCTCATCACGATC	621	Dallenne et al., 2010
bla KPC	F- CATTCAAGGGCTTTCTTGCTGC R-ACGACGGCATAGTCATTTGC	498	Dallenne et al., 2010
blaOXA-48	F-GCTTGATCGCCCTCGATT R-GATTTGCTCCGTGGCCGAAA	281	Dallenne et al., 2010
bla VIM	F- GGTGTTTGGTCGCATATCGCAA R- ATTCAGCCAGATCGGCATCGGC	502	Dallenne et al., 2010
bla IMP	F-TCGTTTGAAGAAGTTAACG	568	Dallenne et al., 2010

	R- ATGTAAGTTTCAAGAGTGATGC		

Chapter 4: Result & Observation

4.1: Isolation of *Klebsiella pneumoniae*

Tolal 50 samples were collected from the pre-selected sample collecting sites during the period of February 2023 to June 2023. Among those 50 samples, 12 samples were collected from the wastewater effluent of the dedicated hospitals and other 38 samples were collected from the adjacent community households of those hospitals. From these 50 samples, a total of 177 (85 isolates from hospital wastewater and 92 isolates from community water) randomly selected (by observing colony morphology) isolates was taken and a total of 119 isolates were PCR confirmed as the *Klebsiella pneumoniae* from which 63 isolates were from hospital wastewater and 56 isolates were from community water. Its noticeable that, more than 67% of the total sample size was confirmed as *K. pneumoniae.* The isolates were taken by observing the colonial morphology of the isolates in KPC media and McConkey Agar media.

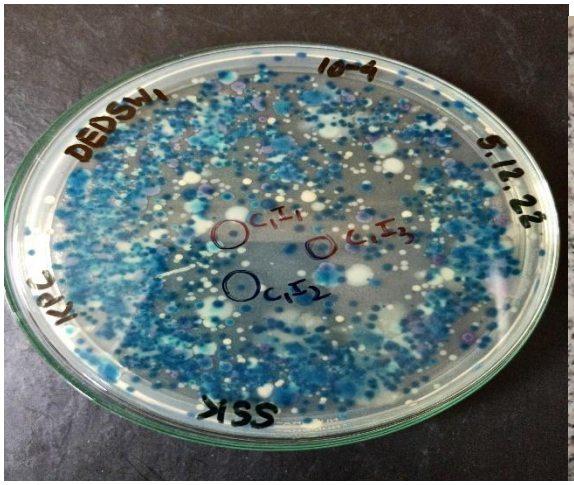


Fig 4.1.1: spread result of Kpn in KPC media

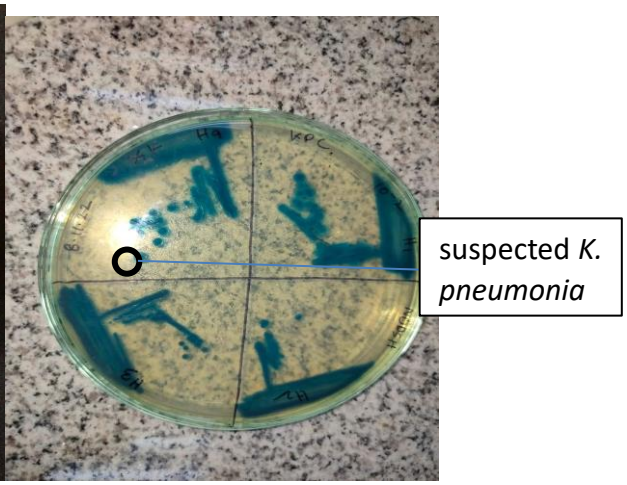
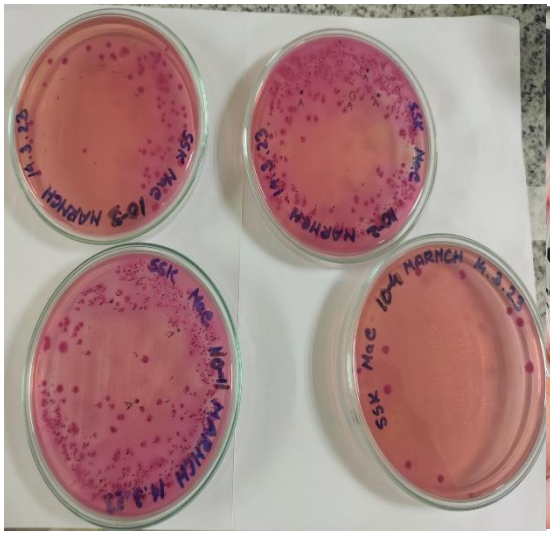


fig 4.1.2: streaking of Kpn in KPC media



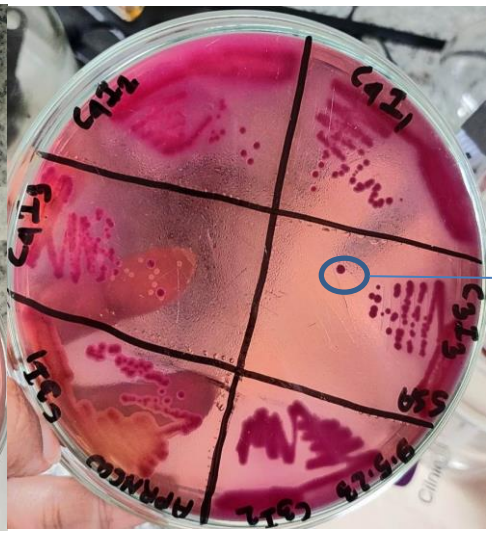


Fig 4.1.3: spread result of Kpn in MacConkey fig4.1.4: streaking of Kpn in MacConkey

4.2: PCR-based identification of *Klebsiella pneumoniae*

After finishing PCR and gel electrophoresis successfully, the gel containing amplified products was taken under the UV illuminator and result was observed by comparing the band size with ladder and positive controls. An isolate was considered as confirmed Kpn when it gave the expected band size. In the following figure, the gel electrophoresis result of PCR-amplified products under UV illuminator is demonstrated:

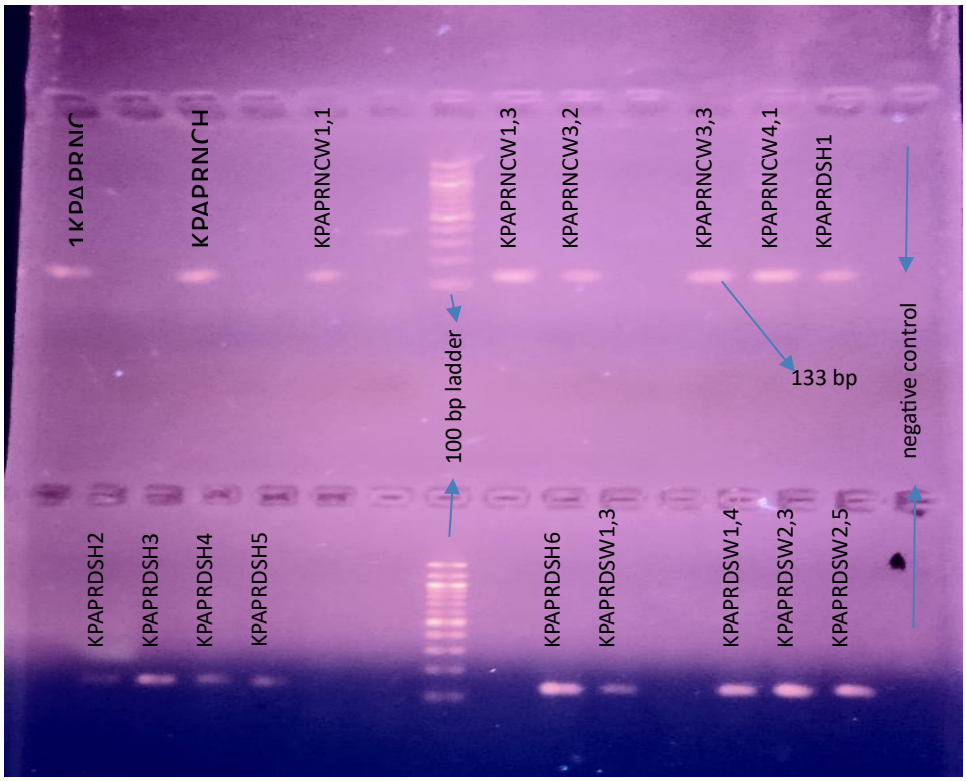


Fig 4.2.1: PCR result of detecting *K. pneumoniae* using 100 bp ladder

4.3 Distribution of *Klebsiella pneumoniae* isolates: month-wise

One of the measure study parts of the research was to observe the tendency of finding *K. pneumoniae* throughout the period to see in which months the isolates were found more. Our time duration was February 2023 to June 2023. Initially selected isolates of *K. pneumoniae* were taken for PCR assay and then confirmed. It has been found that, March has the highest success in isolating Kpn which is around 30%. A total of 36 isolates were being confirmed as *K. pneumoniae.* Additionally, in the month May, PCR confirmed isolates were 31 which is almost 26% of the total PCR confirmed isolates. Moreover, in February, total 24 isolates were confirmed as *K. pneumoniae* which is almost 20% of the total PCR confirmed isolates. Again, in April, 17 isolates were confirmed, and it is almost 14% of the total confirmed isolates (119 isolates). Lastly, in June, only 1 sample was collected and from that sample (both hospital and community) 11 isolates were confirmed which is 9% of the total confirmed isolates.

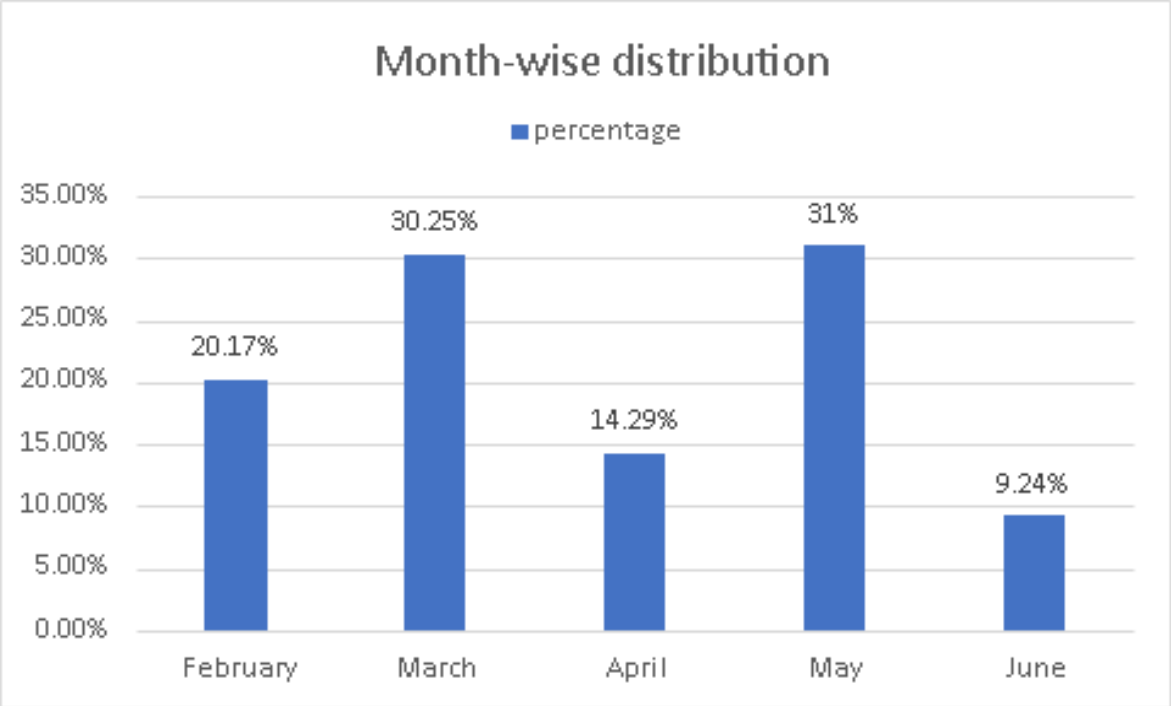


Figure 4.3.1: Month-wise distribution of PCR confirmed *K. pneumoniae*

4.4 Distribution of *K. pneumoniae*: based on the sampling sites

The study was designed with three dedicated sample collecting sites (both hospital and community households) located in Dhaka district. The sample collecting sites were DNCC Dedicated Covid-19 Hospital (DNCC-DCH), Mohakhali, Dhaka-1212, Dhaka Shishu (Children) Hospital (DSH), Shaymoli-1207 and National Institute of Cancer Research & Hospital (NICRH). These places were chosen for the accessibility of the mass people.

After observing the data, it is observed that highest isolates were obtained from Dhaka Shishu (Children) Hospital (DSH) which is 42% (50 isolates) of the total confirmed isolates. Then comes the National Institute of Cancer Research & Hospital (NICRH) from where second highest isolates were obtained which is 37% (44 isolates). Then, from DNCC Dedicated Covid-19 Hospital (DNCC-DCH), total 25 isolates were found which is 21% of total confirmed isolates. From DNCC Dedicated Covid-19 Hospital (DNCC-DCH) less samples were collected that is why the percentage of positive isolates are less from this site. Otherwise, every site has almost similar chances of finding *K. pneumoniae.*

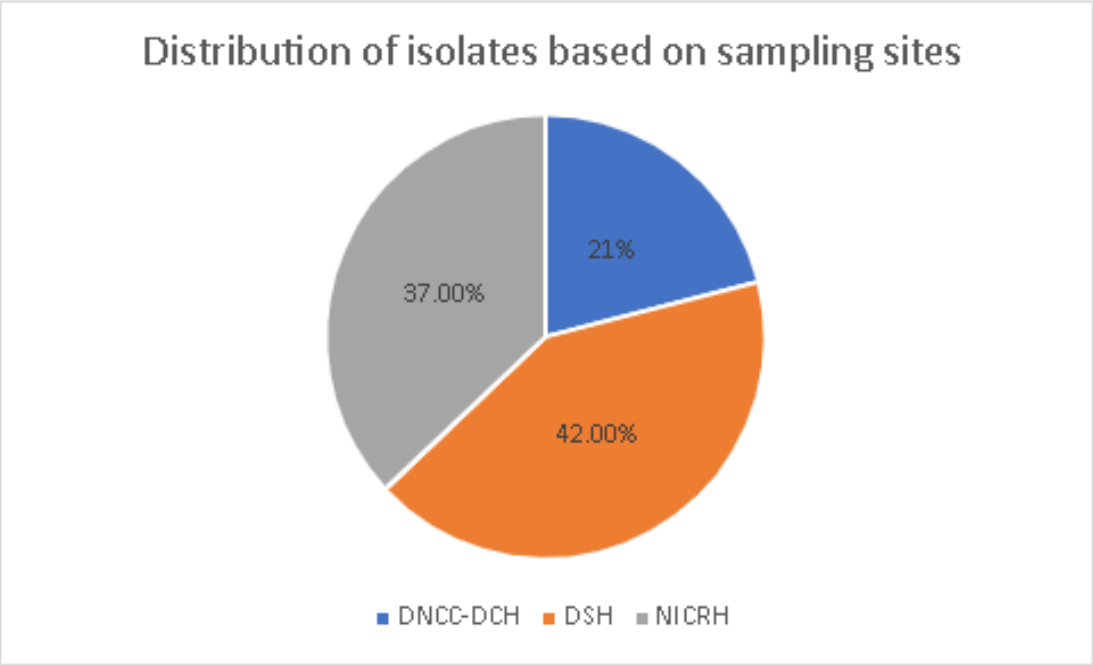


Figure 4.4.1: sampling sites-wise distribution of *K. pneumoniae*

4.5 Antimicrobial Susceptibility Test – Result

After 18-24 hours incubation period, the MHA plates with target isolates and antibiotic dicks were observed and zones were measured in millimeter. Then, the interpretation was done by following CLSI guideline whether the isolate is resistant, intermediate or sensitive. The AST result on MHA media is represented by:

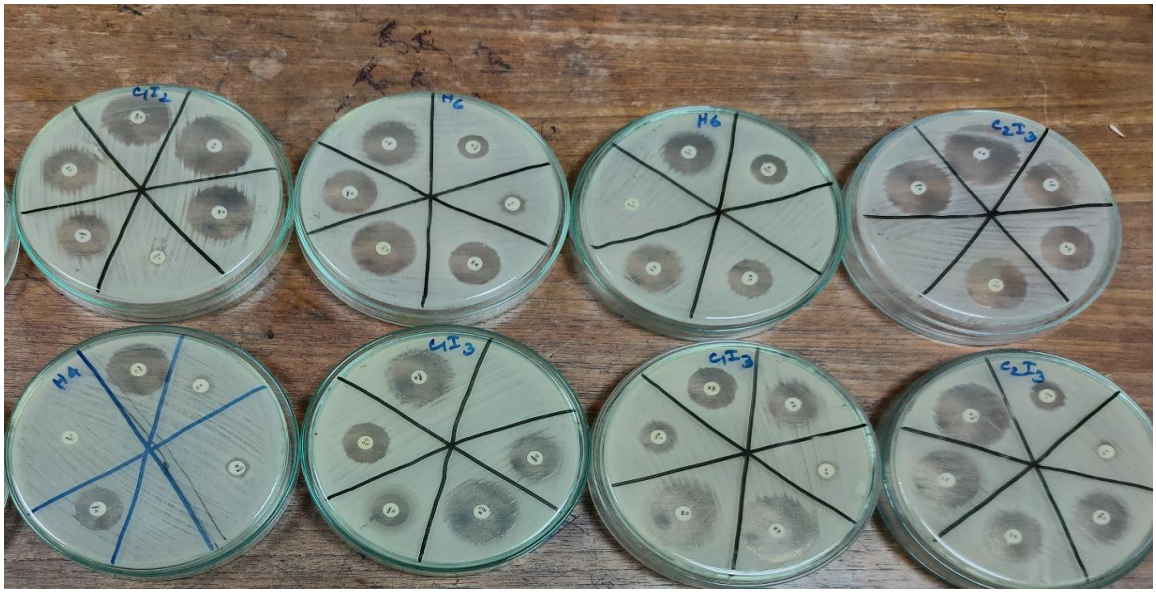


Figure 4.5.1: Antibiotic susceptibility test of *K. pneumoniae*

4.5.1 Antimicrobial resistance pattern of total isolates

After analyzing the data of the interpretation of AST, it is observed that *K. pneumoniae* showed highest resistance to Erythromycin from the macrolides group which is 98%. Almost all the isolates were resistant to Erythromycin. Other 2% were intermediate, so *K. pneumoniae* is 0% sensitive to erythromycin. The second highest resistance the isolates showed was to cefixime from the 3rd Gen cephalosporins group which is 71%. The isolates showed the lowest resistance to gentamicin and amikacin from the aminoglycoside group which are 10% and 6% respectively. The isolates have second highest sensitivity towards imipenem (83%), doxycycline (11%) and norfloxacin (87%). On the other hand, the isolates showed a moderate resistance to ceftriaxone (28%), cefepime (24%), amoxiclav (16%) and aztreonam (14%).

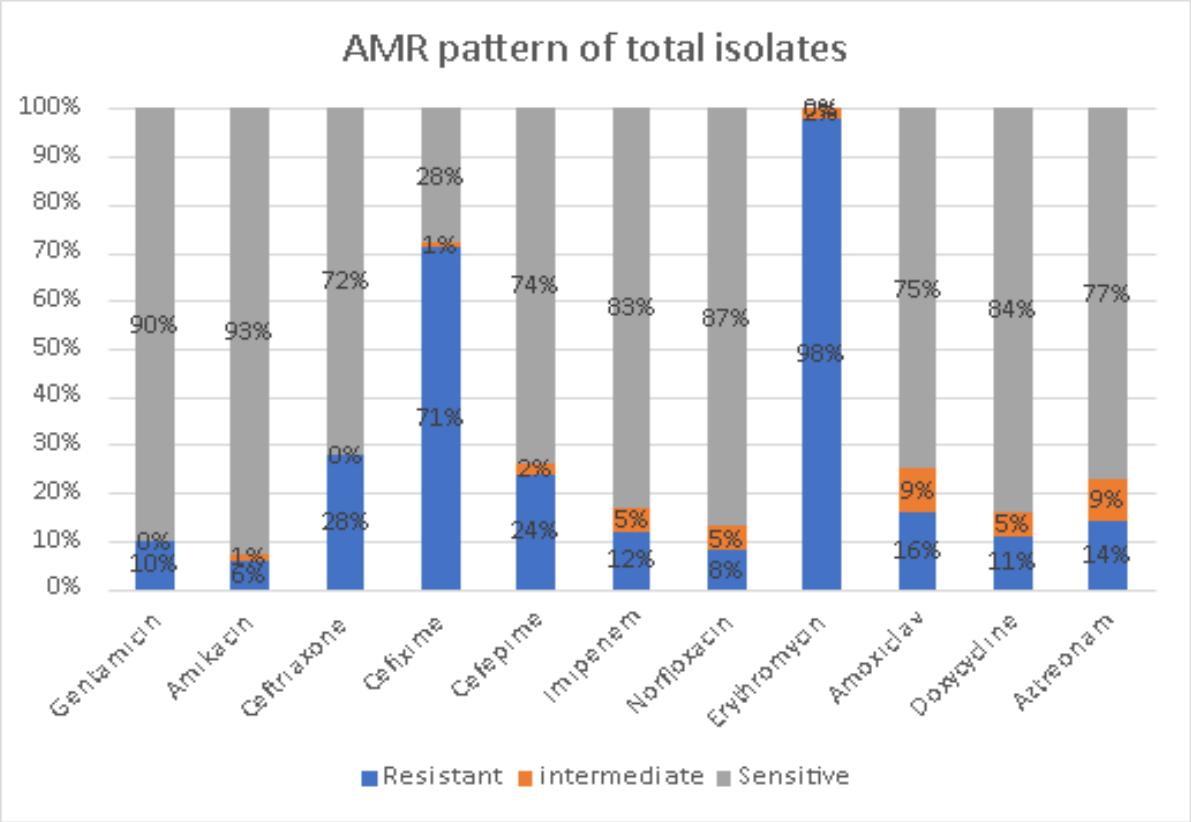


Figure 4.5.1: graphical representation of antimicrobial resistance pattern of total isolates

4.5.2 Antimicrobial resistance pattern in isolates of Hospital effluents

While looking at the AST datasheet of hospital effluents, it is highlighted that again the isolates retrieved from hospital effluents were highly resistant to erythromycin which is 98% of the total *K. pneumoniae* isolated from hospital wastewater. Additionally, the isolates have shown an attractive resistance to cefepime which is 83%. Moreover, second highest resistance has been observed in ceftriaxone (44%) and cefepime (41%). However, gentamicin and amikacin have the highest sensitivity towards *K. pneumoniae.*

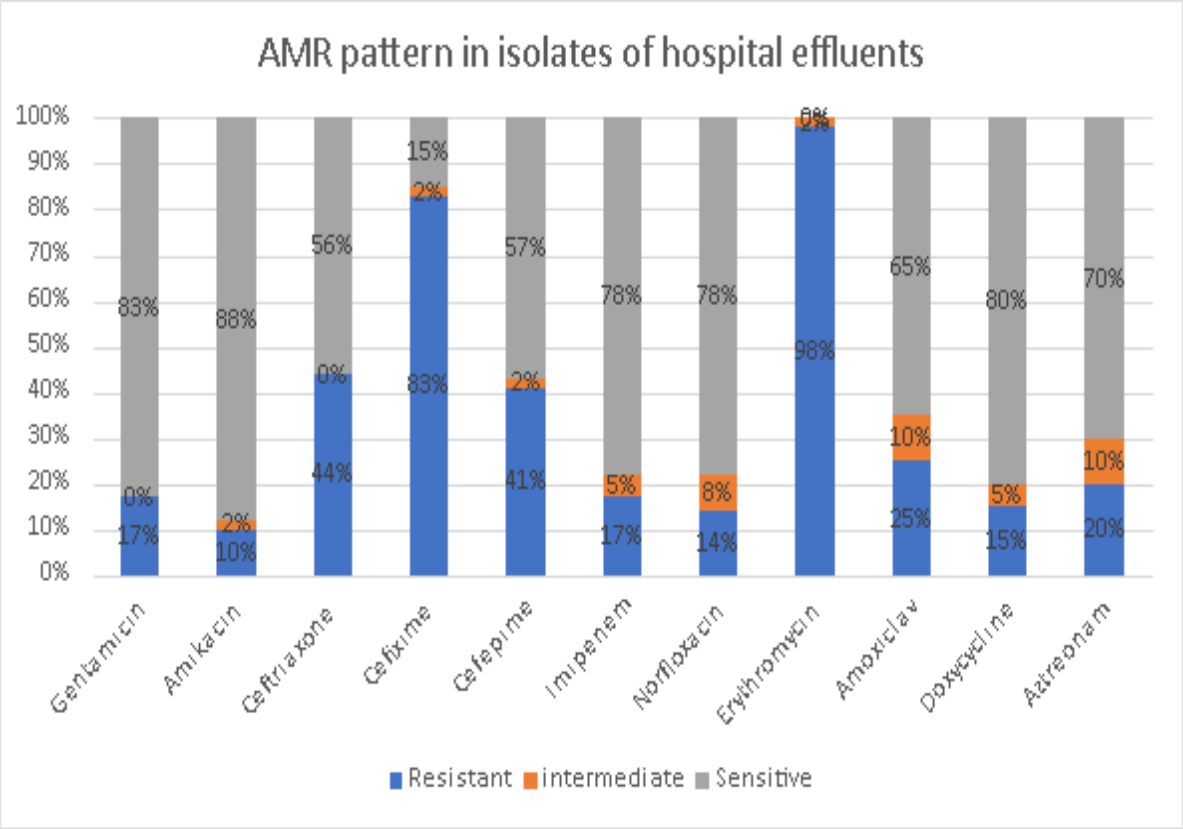


Figure 4.5.2: graphical representation of antimicrobial resistance pattern in isolates of Hospital effluents

4.5.3 Antimicrobial resistance pattern in isolates of Hospital adjacent Communities

By analyzing the data, it is observed that, the isolates showed 0% resistance to gentamicin and amikacin. Isolates obtained from community water were highly resistant to erythromycin (98%) and cefixime (71%). Otherwise, the isolates were very less resistant and highly susceptible to the antibiotics which is between 0% to 7%.

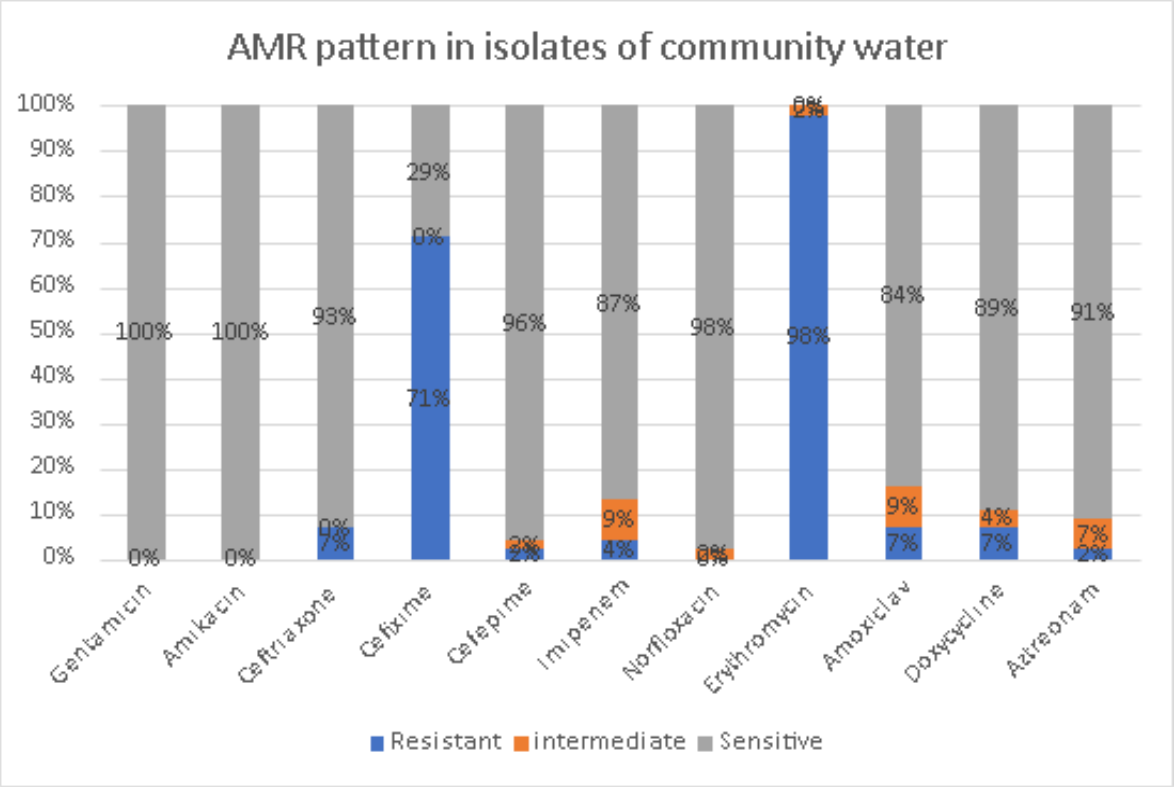


Figure 4.5.3: graphical representation of antimicrobial resistance pattern in isolates of Hospital adjacent communities

4.5.4 Comparative analysis of AMR pattern between the isolates of Hospital wastewater and the adjacent communities of the respective hospitals

The research was conducted to study and compare the pathogenicity of the bacteria found in hospital effluent and the water from the nearby community households of the hospital. Three hospitals from Dhaka city were selected for the study and the hospitals are full of mass people, patients and wastages produced from the hospital. As the locations were the hospital sewerage, it was assumed the isolates obtained from the hospital effluents are more pathogenic and resistant to antibiotic. After analyzing the data, it is considerably true that the isolates from hospital wastewaters are more pathogenic and show more antimicrobial resistance than those of community water. It is significantly noticeable that hospital isolates have shown higher resistance to all the antibiotics than the isolates of the community water. Among those, the isolates of hospital effluents and community water have a huge difference in AMR patter for the antibiotic ceftriaxone and cefepime. For ceftriaxone, isolates of hospital effluents have shown 44% resistance and isolates of community water have shown only 7% resistance. On

the other hand, for cefepime, isolates of hospital effluents have shown 41% resistance and isolates of community water have shown only 2% resistance.

However, both the isolates from hospital effluents and community water have shown the same resistance pattern for erythromycin which is the highest percentage of resistance (98%).

Table 4.5.4: Comparative analysis of AMR pattern between the isolates of Hospital wastewater and the isolates of Hospital adjacent communities.

Antibiotic	Hospital Resistant	Community Resistant	Hospital Intermediate	Community Intermediate	Hospital Sensitive	Community sensitive
Gentamicin	17%	0%	0%	0%	83.00%	100%
Amikacin	10.00%	0%	2%	0%	88.00%	100%
Ceftriaxone	44%	7%	0%	0%	56%	93%
Cefixime	83%	71%	2%	0%	15%	29%
Cefepime	41.00%	2%	2%	2%	57.00%	96%
Imipenem	17.00%	4%	5%	9%	78%	87%
Norfloxacin	14%	0%	8%	2%	78%	98%
Erythromycin	98%	98%	2%	2%	0%	0%
Amoxiclav	25%	7%	10%	9%	65%	84%
Doxycycline	15%	7%	5%	4%	80%	89%
Aztreonam	20%	2%	10%	7%	70%	91%

4.6: AMR-gene testing

4.6.1: Finding from the AMR-gene test

After confirming the isolates as *K. pneumoniae* through PCR assay, 17 specifically selected isolates were chosen for the AMR-gene test. Those isolates were chosen based on their resistance pattern to the antibiotics from the beta-lactam. These isolates were chosen from those isolates which showed resistance to the antibiotics from carbapenem and cephalosporin (3rd generation and 4th generation) group, for example, imipenem, cefepime, ceftriaxone, cefixime.

The result of this test was obtained through gene specific PCR assay. One of those PCR results is given bellow.

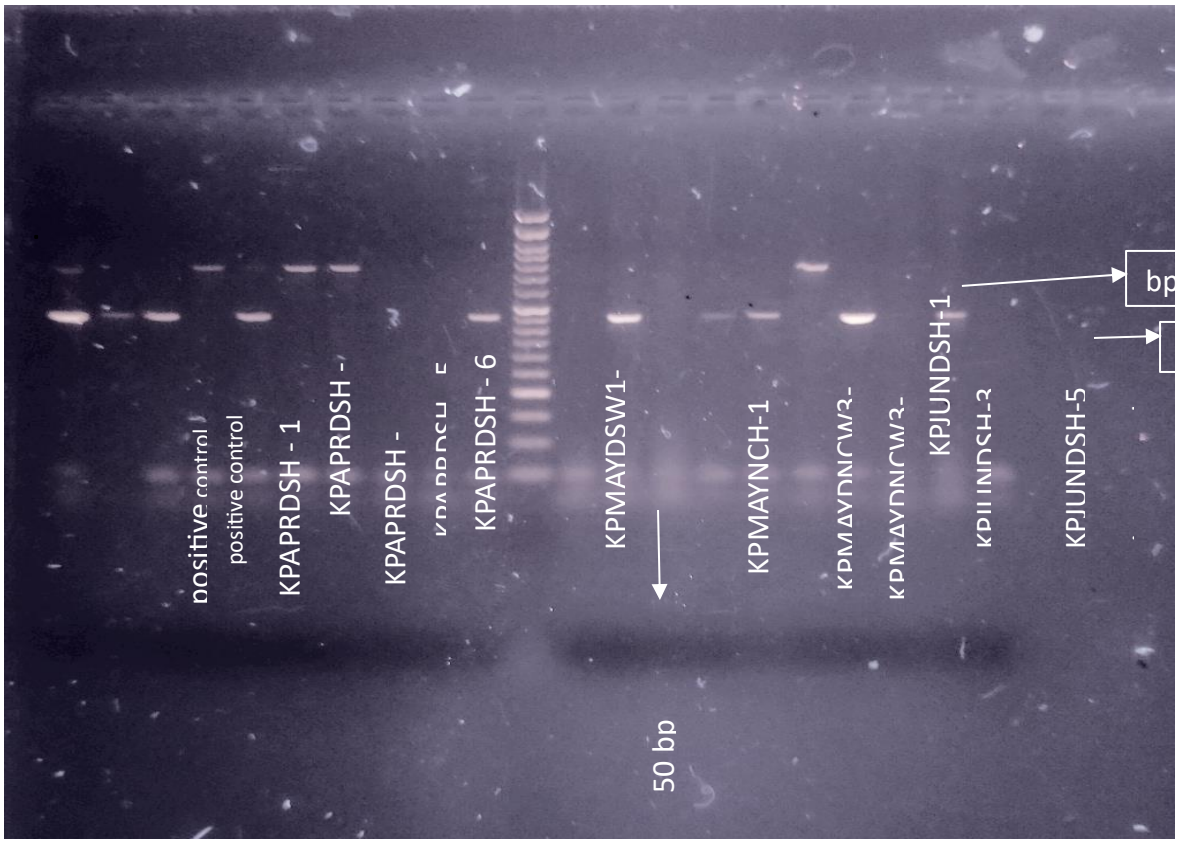


Figure 4.6.1: result of the multiplex PCR of bla-CTXM and bla-SHV

4.6.2: Result analysis of AMR-genes:

Total eight genes were tested for the 17 isolates from which there was no trace of *bla*$_{KPC}$, *bla*$_{OXA48}$, *bla*$_{VIM}$, *bla*$_{IMP}$. The percentage of the presence of these genes are among those selected isolates. On the other hand, *bla*$_{CTXM}$ is present in the higher amount in these isolates. In total 8 isolates, *bla*$_{CTXM}$ gene was found which is 40% of those chosen isolates. Moreover, *Bla*$_{SHV}$, *Bla*$_{TEM}$, *Bla*$_{NDM}$ all these three genes were found in 4 isolates each. That means, these genes are present in almost 20% of the carbapenem and ESBL resistant isolates.

The result interpretation is given bellow:

Table 4.6.2: result interpretation of AMR-gene

Name of AMR-Gene	Number of MR-Gene found	percentage of AMR gene
***Bla*$_{CTXM}$**	**8**	40%
***Bla*$_{SHV}$**	**4**	20%

***Bla*TEM**	**4**	20%
***Bla*NDM**	**4**	20%
***Bla*VIM**	**0**	0%
***Bla*KPC**	**0**	0%
***Bla*IMP**	**0**	0%
***Bla*OXA48**	**0**	0%

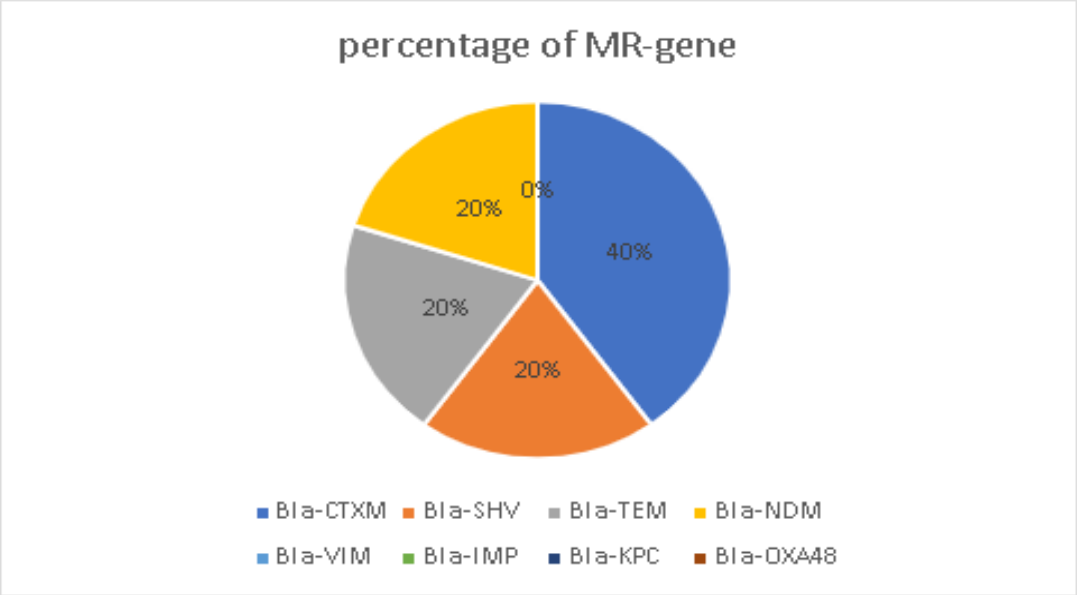


Figure 4.6.2: graphical representation of the results of the AMR-gene tests

Chapter 5: discussion

5.1: Result based discussion:

Antimicrobials which include antibiotics, antiviral, antifungals and antiparasitic are commonly used to treat and prevent infections. (Prestinaci et al., 2015) Antimicrobial resistance is now a global threat as bacteria, virus, fungi and parasites are also evolving or adapting or mutating over the time and no longer responding to the medicines. As a result, it is becoming more difficult to treat the infections and the microorganisms are continuously resisting the medicines. Consequently, the diseases are spreading and causing severe infections and increasing death. (Prestinaci et al., 2015) Around 1.27 million people died worldwide associated with nearly 5 million deaths in 2019 because of the antimicrobial resistant microorganisms. Microorganisms do not need to be resistant to all the antibiotics, it can be life threatening even if it is showing resistance to only one antibiotic as in many treatments, some major antibiotics are the key treatment and doctors must depend on that. (CDC, 2022) Sometimes, antibiotic resistant bacterial infections need second- or third-line treatment which is quite hard on the patients as it may cause organ failure including extension of the time-period of the treatment. (CDC, 2022) *K. pneumoniae* is one of those bacteria which is changing rapidly and showing resistance to the antibiotics. Resistance found in *K. pneumoniae* to carbapenem antibiotics has spread to all over the world and in many countries, carbapenem antibiotics do not work in almost more than half of the patients treated for *K. pneumoniae* infections due to the resistance. (Prestinaci et al., 2015) Hence, the rate of resistance to ciprofloxacin which is an antibiotic commonly used to treat urinary tract infections, varied from 4.1% to 79.4% for *K. pneumoniae* in countries reporting to the Global Antimicrobial Resistance and Use Surveillance System (GLASS) which is very alarming. (*Antimicrobial resistance*, n.d.) Again, the global drug resistance rate of *K. pneumoniae* has reached almost 70%, and the infection-related fatality rate has also reached from 40% to 70% which is why in recent years, multiple-drug resistance (MDR) *K. pneumoniae* and carbapenem-resistant *K. pneumoniae* (CRKP) have been a major global public health threat. (Li et al., 2022)

One of the most important concerns of our study is to distinguish between the AMR patterns of the isolates from hospital effluents and the isolates from the adjacent community water. Hospitals were our prime location because Healthcare Associated Infections are another threat for human in this modern world. HCAIs are the infections that can cause when people go to healthcare facilities to treat other diseases. The US Center for Disease Control and Prevention

states that almost 1.7 million hospitalized patients annually get HCAIs while being treated for other health issues and that more than 98,000 patients which means one in 17 die due to these infections. (Haque et al., 2018) Additionally, a study revealed that for every 20 patients hospitalized, at least one acquired an HCAI. (Haque et al., 2018) On the other hand, *K. pneumoniae* and the *Acinetobacter* species were extremely resistant to multiple antimicrobials so that the lack of new anti-microbials increases the huge burden in Europe. (Haque et al., 2018) Again, an analysis regarding HCAIs in Southeast Asian countries (Brunei, Myanmar, Cambodia, East Timor, Indonesia, Laos, Malaysia, the Philippines, Singapore, Thailand, and Vietnam) found an overall prevalence rate of 9.1% and the most common microorganisms are *P. aeruginosa*, the *Klebsiella* species, and *Acinetobacter baumannii*. (Haque et al., 2018) So, *K. pneumoniae* plays an important role in HCAIs. *K. pneumoniae is* mutating very rapidly and become carbapenem and ESBL resistant. In our country, hospital effluents get released in the environment without any treatment. Both dry and liquid wastes of the hospitals are disposed every day without taking any precautions. As a result, there is a high risk of HCAIs in the hospitals. Moreover, our target organism is highly prevalent and resistant to antibiotics. So, it has greater chance of causing HCAIs.

Our study was conducted by focusing on the hospital areas and its adjacent communities located in the Dhaka North city-corporation. Our sample collecting sites for the study were DNCC Dedicated Covid-19 Hospital, Mohakhali, Dhaka-1212, National Institute of Cancer Research & Hospital (NICRH), and Dhaka Shishu (Children) Hospital, Shaymoli-1207 and their adjacent communities. The isolates of *K. pneumoniae* were labelled as hospital effluent's isolates and community water isolates. After analyzing the data from February 2023 to June 2023 (which is our own data) it is found that, 67% of the total isolates were confirmed as *K. pneumoniae.* The isolates were confirmed as the target organism by doing PCR.

Our study has found that large number of isolates showed resistance to multiple antibiotics. Among hospitals isolates, the isolates showed resistance to all the 12 antibiotics. The isolates from hospital effluents showed 44% resistance to ceftriaxone and most importantly, 84% resistance to cefixime which is remarkable. According to the research of WHO world region, in African region, 27.6% *K. pneumoniae* are resistant to 3rd generation cephalosporin and in American region, 36% isolates showed resistance. (Prestinaci et al., 2015) One study in Addis Ababa has found that 29.6% isolates of *K. pneumoniae* were resistant to one or more than one carbapenems. (Awoke et al., 2022) Our research also found 41% resistance to cefepime (4th

generation cephalosporin) Another study was conducted in Bangladesh, where the research shows that, 56% K. pneumoniae strains were multi-drug resistant (Hussain et al., 2023) where in our study, 39% (23 out of 59 isolates) were MDR. In that research, they found that, only 9% if the isolates were carbapenem (imipenem) resistant where our study found that 12% of the isolates are carbapenem(imipenem) resistant which is very similar. (Hussain et al., 2023) So, the isolates are showing an alarming amount of resistance to cephalosporins which means they are showing resistance to beta-lactams.

On the other hand, among the isolates of community water, the isolates showed zero resistance to gentamycin and amikacin. Otherwise, the isolates showed resistance to the other 10 antibiotics as well. The isolates of community water showed the highest resistance to the cefixime. However, both the isolates from hospital effluents and community water showed 0% susceptibility and 98% resistance to erythromycin.

Most significantly, the isolates from hospital effluents showed more resistance than the isolates of community water. So, it should be taken seriously that, hospital effluents should be disinfected and treated properly before disposing those in the environment, otherwise Càis will increase, and this highly prevalent and AMR *K. pneumoniae* will be spread to the environment and community.

Another part of the study was to identify the responsible genes for showing AMR. Total eight genes were selected and tested which are: five genes bla_{NDM}, bla_{KPC}, bla_{OXA48}, bla_{VIM} and bla_{IMP} which are the gene responsible for carbapenemase production and bla_{CTXM}, bla_{SHV} and bla_{TEM} which are responsible for ESBL production. From these eight genes, bla_{CTXM}, bla_{SHV} and bla_{TEM} were found and from carbapenemase producing gene, bla_{NDM} was found. After analyzing the AST data, 17 isolates were chosen for AMR gene test and from those isolates, 40% isolates have bla_{CTXM}, 20% isolates have bla_{SHV}, 20% isolates have bla_{TEM} and other 20% isolates have bla_{NDM}. In research from Jilin province, China, it is found that, 34.4% isolates carried $bla_{\text{CTXM,}}$ 32.26% isolates carried bla_{SHV} and 56.99% isolates carried bla_{TEM}. (Liu et al., 2023) They also found 91.4% bla_{NDM} which was not present in our isolates. So, it is confirmed that ESBL and carbapenemase producing genes are present in the isolates and that is why they are carbapenem and beta-lactam or ESBL resistant. So, it is necessary to prevent AMR immediately.

5.2: Limitations of our study:

All three sample collecting sites of our study were in Dhaka North City Corporation, so the locations do not cover the whole Dhaka city. So, our study cannot be a complete representation of Dhaka city as the locations do not cover South City Corporation. Moreover, among those three hospitals, DNCC dedicated COVID-19 hospital was not that much active in service for last few months compared to the other two hospitals. On the other hand, our study only focused on microbiological analysis and did not do any chemical and physical evaluation of the hospital effluents to find out the other carcinogens present in this water or did not analyze if there is any connection between the physical/ chemical condition of the wastewater or community water and the AMR of the isolates.

Chapter 6

Conclusion

Klebsiella pneumoniae is one of the major bacteria which is highly evolving and resisting multiple antibiotics. It has become a great threat for public health because of its carbapenem and beta-lactams resistance. Carbapenem resistant *K. pneumoniae* or CRKP has caught everyone's attention for its high prevalence. Carbapenems are strong antibiotics with a large antibacterial spectrum. Carbapenems are much preferable for the treatment of serious Enterobacteriaceae bacterial infections. But, recently, the emergence and prevalence of CRKP have caused a serious threat to patients with low immune function and have become a distinct risk factor leading to the death of patients with nosocomial infections. (Li et al., 2022) In our study the isolates from hospital effluents have shown resistance to all the antibiotics and the isolates from the community water has shown resistance to almost 10 antibiotics.

In our country, very less or zero precautions are being taken to prevent HCAIs. No proper arrangements are available to treat the hospital effluent and dry wastages properly before disposal. As a result, the bacteria and other microorganisms are getting released to the environment. The bacteria are evolving with antibiotic resistant gene (ARGs). There are some basic reasons behind this rapid AMR pattern of the bacteria which are:

- In our country, people overuse the antibiotic, where the antibiotics are not necessary, people intake antibiotic there also without a prescription of the doctor.
- People misuse the antibiotics, whether they do not take the antibiotic as prescribed, or they do not continue to take the antibiotics or do not complete the full course. As a result, the bacteria start to reproduce and mutate.
- Sometimes bacteria in the animal body can also be resistant as the overuse of antibiotics in livestock.
- Sometimes the genetic makeup of the bacteria can change by its own and become resistant.
- The contagious antibiotic resistant bacterial infection can be spread from one person to another person.
- Poor facilities in infection control sector of the hospitals can be another reason.
- Use of chemicals in agriculture, soil, foods and other manufactured products.
- Unplanned dumping of medical instruments and expired medicines.
- Improper hygiene practices in health care centers.

We must work frequently and massively to mitigate HCAIs and AMR which is a long-term process. There are some suggested steps which we can follow to prevent these problems:

- We must take antibiotic only when it is necessary. Doctors must suggest patients antibiotics very carefully. One must not take others prescribed antibiotics.
- A specific antibiotic must be provided which will target her bacteria and will be beneficial for the patient to cure illness.
- The course of the antibiotics must be completed.
- Hygiene level of the hospitals must be in high level to avoid contamination.
- The hospital wastages must be treated and then disposed in a proper way.
- Viral infections must not be treated with the antibiotics.

Chapter 7: Reference

1. Wikipedia contributors. (2023, June 8). *Klebsiella pneumoniae* e. Wikipedia, The Free Encyclopedia. https://en.wikipedia.org/w/index.php?title=Klebsiella_pneumoniae&oldid=1159177632

2. Klebsiella infections. (2023, June 13). Medscape.com. https://emedicine.medscape.com/article/219907-overview?form=fpf

3. Brabb, T., Newsome, D., Burich, A., & Hanes, M. (2012). Infectious Diseases. In The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents (pp. 637–683). Elsevier.

4. Aryal, S. (2015, November 30). Biochemical Test and Identification of *Klebsiella pneumoniae* e. Microbiology Info.com; Microbiology Info. https://microbiologyinfo.com/biochemical-test-and-identification-of-klebsiella-pneumon iae/

5. Dey, H., Vasudevan, K., Dasegowda, K. R., Rambabu, M., Cn, P., & Doss, G. P. (2022). An integrated gene network analysis to decode the multi-drug resistance mechanism in *Klebsiella pneumoniae* e. Microbial Pathogenesis, 173(105878), 105878. https://doi.org/10.1016/j.micpath.2022.105878

6. Zhu, J., Wang, T., Chen, L., & Du, H. (2021). Virulence Factors in Hypervirulent *Klebsiella pneumoniae* e. Frontiers in Microbiology, 12. https://doi.org/10.3389/fmicb.2021.642484

7. Lawlor, M. S., Hsu, J., Rick, P. D., & Miller, V. L. (2005). Identification of*Klebsiella pneumoniae* evirulence determinants using an intranasal infection model: <I>*Klebsiella pneumoniae* e</i>intranasal STM. Molecular Microbiology, 58(4), 1054–1073. https://doi.org/10.1111/j.1365-2958.2005.04918.x

8. *Klebsiella infections*. (2023, June 13). Medscape.com. https://emedicine.medscape.com/article/219907-overview?form=fpf

9. Ahmadi, M., Ranjbar, R., Behzadi, P., & Mohammadian, T. (2022). Virulence factors, antibiotic resistance patterns, and molecular types of clinical isolates of *Klebsiella pneumoniae e*. *Expert Review of Anti-Infective Therapy*, *20*(3), 463–472. https://doi.org/10.1080/14787210.2022.1990040

10. Ashurst, J. V., & Dawson, A. (2023). *Klebsiella pneumoniae* . StatPearls Publishing.

11. Dey, H., Vasudevan, K., Dasegowda, K. R., Rambabu, M., Cn, P., & Doss, G. P. (2022). An integrated gene network analysis to decode the multi-drug resistance mechanism in *Klebsiella pneumoniae* e. *Microbial Pathogenesis*, *173*(105878), 105878. https://doi.org/10.1016/j.micpath.2022.105878

12. Khan, H. A., Ahmad, A., & Mehboob, R. (2015). Nosocomial infections and their control strategies. *Asian Pacific Journal of Tropical Biomedicine*, *5*(7), 509–514. https://doi.org/10.1016/j.apjtb.2015.05.001

13. Le, T.-H., Ng, C., Chen, H., Yi, X. Z., Koh, T. H., Barkham, T. M. S., Zhou, Z., & Gin, K. Y.-H. (2016). Occurrences and characterization of antibiotic-resistant bacteria and genetic determinants of hospital Wastewater in a tropical country. *Antimicrobial Agents and Chemotherapy*, *60*(12), 7449–7456. https://doi.org/10.1128/aac.01556-16

14. Hocquet, D., Muller, A., & Bertrand, X. (2016). What happens in hospitals does not stay in hospitals: antibiotic-resistant bacteria in hospital wastewater systems. *The Journal of Hospital Infection*, *93*(4), 395–402. https://doi.org/10.1016/j.jhin.2016.01.010

15. An, R., Qi, Y., Zhang, X.-X., & Ma, L. (2023). Xenogenetic evolutionary of integrons promotes the environmental pollution of antibiotic resistance genes — Challenges, progress and prospects. *Water Research*, *231*(119629), 119629. https://doi.org/10.1016/j.watres.2023.119629

16. Domingues, S., da Silva, G. J., & Nielsen, K. M. (2012). Integrons: Vehicles and pathways for horizontal dissemination in bacteria. *Mobile Genetic Elements*, *2*(5), 211–223. https://doi.org/10.4161/mge.22967

17. Al Aukidy, M., Al Chalabi, S., & Verlicchi, P. (2017). Hospital wastewater treatments adopted in Asia, Africa, and Australia. In *The Handbook of Environmental Chemistry* (pp. 171–188). Springer International Publishing.

18. Vanhooren, P. T., De Baets, S., Bruggeman, G., & Vandamme, E. J. (1999).

19. Wikipedia contributors. (2023, June 8). *Klebsiella pneumoniae e*. Wikipedia, The Free Encyclopedia. https://en.wikipedia.org/w/index.php?title=Klebsiella_pneumoniae&oldid=1159177632

20. Sydow, K., Eger, E., Schwabe, M., Heiden, S. E., Bohnert, J. A., Franzenburg, S., Jurischka, C., Schierack, P., & Schaufler, K. (2022). Geno- and phenotypic characteristics of a *Klebsiella pneumoniae* e ST20 isolate with unusual colony morphology. *Microorganisms*, *10*(10), 2063. https://doi.org/10.3390/microorganisms10102063

21. Lawlor, M. S., Hsu, J., Rick, P. D., & Miller, V. L. (2005). Identification of*Klebsiella pneumoniae e*virulence determinants using an intranasal infection model: <I>*Klebsiella pneumoniae* e</i>intranasal STM. *Molecular Microbiology*, *58*(4), 1054–1073. https://doi.org/10.1111/j.1365-2958.2005.04918.x

22. Highsmith, A. K., & Jarvis, W. R. (1985). *Klebsiella pneumoniae e:*Selected Virulence Factors that Contribute to Pathogenicity. *Infection Control: IC*, *6*(2), 75–77.

https://doi.org/10.1017/s0195941700062640

23. Strakova, N., Korena, K., & Karpiskova, R. (2021). *Klebsiella pneumoniae* e producing bacterial toxin colibactin as a risk of colorectal cancer development - A systematic review. *Toxicon: Official Journal of the International Society on Toxinology*, *197*, 126–135. https://doi.org/10.1016/j.toxicon.2021.04.007

24. Al Aukidy, M., Al Chalabi, S., & Verlicchi, P. (2017). Hospital wastewater treatments adopted in Asia, Africa, and Australia. In *The Handbook of Environmental Chemistry* (pp. 171–188). Springer International Publishing.

25. Korzeniewska, E., & Harnisz, M. (2013). Extended-spectrum beta-lactamase (ESBL)-positive Enterobacteriaceae in municipal sewage and their emission to the environment. *Journal of Environmental Management*, *128*, 904–911. https://doi.org/10.1016/j.jenvman.2013.06.051

26. *Revista de la Academia Colombiana de Ciencias Exactas, Físicas y Naturales - Home Page*. (n.d.). Org.Co. Retrieved August 21, 2023, from http://www.scielo.org.co/scielo.php?script=sci_serial&pid=0370-3908&lng=en&nrm=iso

27. Korzeniewska, E., & Harnisz, M. (2013). Extended-spectrum beta-lactamase (ESBL)-positive Enterobacteriaceae in municipal sewage and their emission to the environment. *Journal of Environmental Management*, *128*, 904–911. https://doi.org/10.1016/j.jenvman.2013.06.051

28. von Wintersdorff, C. J. H., Penders, J., van Niekerk, J. M., Mills, N. D., Majumder, S., van Alphen, L. B., Savelkoul, P. H. M., & Wolffs, P. F. G. (2016). Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.00173

29. An, R., Qi, Y., Zhang, X.-X., & Ma, L. (2023). Xenogenetic evolutionary of integrons promotes the environmental pollution of antibiotic resistance genes — Challenges, progress and prospects. *Water Research*, *231*(119629), 119629. https://doi.org/10.1016/j.watres.2023.119629

30. Austin, B. (1999). AEROMONAS | detection by cultural and modern techniques. In *Encyclopedia of Food Microbiology* (pp. 30–37). Elsevier.

31. Tan, A. (2018, March 13). *What is the function of a Tris buffer in DNA extraction?* Sciencing; Leaf Group. https://sciencing.com/function-tris-buffer-dna-extraction 6370973.html

32. *TBE buffer (Tris-Borate-EDTA buffer) 1x, 5x & 10x*. (2020, April 7). Sharebiology. https://sharebiology.com/tbe-buffer-tris-borate-edta-buffer/

33. *Ethidium bromide - UK*. (n.d.). Retrieved August 9, 2023, from https://www.thermofisher.com/bd/en/home/life-science/dna-rna-purification analysis/nucleic-acid-gel-electrophoresis/dna-stains/etbr.html

34. Harrigan, W. F., & McCance, M. E. (1966). Determination of the number of viable organisms in a sample. In *Laboratory Methods in Microbiology* (pp. 21–29). Elsevier.

35. The Editors of Encyclopedia Britannica. (2023). polymerase chain reaction. In *Encyclopedia Britannica*.

36. De Pietro Crt, M. (2022, February 28). What to know about PCR tests?

37. Tankeshwar, A. (2019, September 13). *Agarose gel electrophoresis: Principle, procedure, results*. Microbe Online. https://microbeonline.com/agarose-gel electrophoresis/

38. Kundu, J., Kansal, S., Rathore, S., Kaundal, M., Angrup, A., Biswal, M., Walia, K., & Ray, P. (2022). Evaluation of ERIC-PCR and MALDI-TOF as typing tools for multidrug resistant *Klebsiella pneumoniae* e clinical isolates from a tertiary care center in India. *PloS One*, *17*(11), e0271652. https://doi.org/10.1371/journal.pone.0271652

39. Awoke, T., Teka, B., Aseffa, A., Sebre, S., Seman, A., Yeshitela, B., Abebe, T., & Mihret, A. (2022). Detection of blaKPC and blaNDM carbapenemase genes among *Klebsiella pneumoniae* e isolates in Addis Ababa, Ethiopia: Dominance of blaNDM. *PloS One*, *17*(4), e0267657. https://doi.org/10.1371/journal.pone.0267657

40. Dallenne, C., Da Costa, A., Decré, D., Favier, C., & Arlet, G. (2010). Development of a set of multiplex PCR assays for the detection of genes encoding important β lactamases in Enterobacteriaceae. *The Journal of Antimicrobial Chemotherapy*, *65*(3), 490–495. https://doi.org/10.1093/jac/dkp498

41. *Antimicrobial resistance*. (n.d.). Who.int. Retrieved August 16, 2023, from https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance 42. CDC. (2022, October 5). *What Exactly is Antibiotic Resistance?* Centers for Disease Control and Prevention. https://www.cdc.gov/drugresistance/about.html 43. Li, Y., Kumar, S., Zhang, L., & Wu, H. (2022). *Klebsiella pneumoniae* and its antibiotic resistance: A bibliometric analysis. *BioMed Research International*, *2022*, 1–10. https://doi.org/10.1155/2022/1668789

42. Loudermilk, E. M., Kotay, S. M., Barry, K. E., Parikh, H. I., Colosi, L. M., & Mathers, A. J. (2022). Tracking *Klebsiella pneumoniae* e carbapenemase gene as an indicator of antimicrobial resistance dissemination from a hospital to surface water via a municipal wastewater treatment plant. *Water Research*, *213*(118151), 118151. https://doi.org/10.1016/j.watres.2022.118151

43. (N.d.). Retrieved September 12, 2023, from http://file:///C:/Users/islam/Downloads/pathogens-03-00743.pdf

44. Liu, M., Zheng, L., Zhu, L., Lu, G., Guo, H., Guan, J., Jing, J., Sun, S., Wang, Y., Wang, Z., Sun, Y., Ji, X., Jiang, B., Liu, J., Zhang, W., & Guo, X. (2023). Characteristics of Carbapenem-resistant *Klebsiella pneumoniae* e in sewage from a tertiary hospital in Jilin Province, China. *PloS One*, *18*(5), e0285730. https://doi.org/10.1371/journal.pone.0285730\

45. Yamagishi, J., Sato, Y., Shinozaki, N., Ye, B., Tsuboi, A., Nagasaki, M., & Yamashita, R. (2016). Comparison of boiling and robotics automation method in DNA extraction for metagenomic sequencing of human oral microbes. *PloS One*, *11*(4), e0154389. https://doi.org/10.1371/journal.pone.0154389

46. Hussain, A., Mazumder, R., Ahmed, A., Saima, U., Phelan, J. E., Campino, S., Ahmed, D., Asadulghani, M., Clark, T. G., & Mondal, D. (2023). Genome dynamics of high-risk resistant and hypervirulent *Klebsiella pneumoniae* e clones in Dhaka, Bangladesh. *Frontiers in Microbiology*, *14*. https://doi.org/10.3389/fmicb.2023.1184196