DETECTION OF EXTENDED SPECTRUM β-LACTAMASE (ESBL) AND CARBAPENEMASE ENCODING *Klebsiella pneumoniae* ISOLATES FROM HOSPITAL EFFLUENT WASTEWATER AND HOSPITAL ADJACENT COMMUNITY TAP WATER IN DHAKA METROPOLITAN CITY

By

Sifat Sarwa Siddique

19126048

Shah Noor

19126015

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 Microbiology.

Microbiology Program

Department of Mathematics and Natural Sciences

School of Data Science

BRAC University

June 2023

Declaration

It is hereby declared that

1. The thesis submitted titled **"Detection Extended Spectrum β-Lactamase (ESBL) and Carbapenemase encoding Klebsiella pneumoniae isolates from Hospital Effluent Wastewater and Hospital Adjacent Community Tap Water in Dhaka Metropolitan City "** is our original work while completing our degree at BRAC University.

2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

3. The thesis does not contain material that has been accepted, or submitted, for any other degree or diploma at a university or other institution.

4. We have acknowledged all main sources of help

Student's Full Name and Signature:

Sifat Sarwa Siddique	**Shah Noor**
19126048	**19126015**

Approval:

The thesis titled **"Detection Extended Spectrum β-Lactamase (ESBL) and Carbapenemase encoding Klebsiella pneumoniae isolates from Hospital Effluent Wastewater and Hospital Adjacent Community Tap Water in Dhaka Metropolitan City "** submitted by

1. Sifat Sarwa Siddique- 19126048

2. Shah Noor- 19126015

has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Microbiology in June 2023

Examining Committee:

Supervisor:
(Member)

_____

Md. Hasanuzzaman
Senior Lecturer,
Department of Mathematics and Natural Sciences
BRAC University

Program Coordinator:
(Member)

_____

Dr. Nadia Sultana Deen
Associate Professor,
Department of Mathematics and Natural Sciences
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, samples from selected venues were collected following all the necessary precautions. All the experiments were done at BRAC University Life Sciences Laboratory. It should also be noted that no animal or human models were used in this study.

Abstract

Klebsiella pneumoniae is one of the most occurring opportunistic microbial species responsible for pneumonia infections as well as nosocomial infections (Cordova-Espinoza et al., 2023). This species of bacteria is mostly found in the environment including soil, surface water, and medical devices (Valenzuela-Valderrama et al., 2019). Among ***Klebsiella pneumoniae***, those strains that produce extended-spectrum beta-lactamases are important pathogens responsible for nosocomial infections which can extend to serious bacteremia and pneumonia (Filis et al., 2021). A global study has shown that around 20% to 80% of ***Klebsiella pneumoniae*** were resistant to first-line antibiotics like cephalosporins, fluoroquinolones, and aminoglycosides back in the 2000s (Pitout et al., 2015). The multi-drug resistance state of ***K. pneumoniae*** has led to the emergence of incurable diseases. In our study, we aimed to find ESBL encoding and Carbapenem-resistant ***Klebsiella pneumoniae*** isolates from the Hospital wastewater and their adjacent community tap water of Dhaka Metropolitan City. From our study of 82 confirmed ***K. pneumoniae*** isolates, 24 isolates were selected based on the phenotypic characteristics of the Antimicrobial Susceptibility Test (AST). Among these 24 isolates, 23.3% were positive for $bla_{\text{CTX-M}}$ and 6.7% were positive for bla_{TEM}, bla_{KPC}, and $bla_{\text{NDM-1}}$ genes respectively. Moreover, for the SHV gene, 56.7% of isolates were positive. This ratio indicates that the bacteria ***K. pneumoniae*** acquired from both the hospital effluent and community tap water retains the ability to resist multiple classes of antibiotics.

Keywords: *Klebsiella pneumoniae***,** Antibiotics, ESBL, Carbapenem, resistant, Hospital, wastewater, Community

Dedication

I, **Sifat Sarwa Siddique**, want to dedicate my Thesis to **My Beloved Parents**, **My Younger Brother, My Childhood Teacher Mamata Saha Miss** and My University Faculties, especially **Md. Hasannuzzan Sir** (Senior Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University) and **Akash Ahmed Sir** (Senior Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences). Without their immense support, good wishes, and encouragement I may not survive and complete this journey. I will always and always will be indebted to them.

I, **Shah Noor,** would like to dedicate this Thesis to **My Dearest Parents** and My University Faculties specially **Md. Hasannuzzan Sir** (Senior Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University) and **Akash Ahmed Sir** (Senior Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University). Their encouragement and well wishes empowered me throughout my Thesis. I will forever be grateful to them for facilitating me with knowledge and wisdom so that I could achieve the epilogue of my bachelor's degree.

Acknowledgment

We would like to begin by expressing our gratitude to Almighty Allah for providing us with the opportunity and strength to complete this research. Additionally, we are grateful for His blessings on our day-to-day lives, good health, and sound mind. We acknowledge our esteemed **Professor A F M Yusuf Haider, Ph.D.,** Professor and Chairperson of the Department of Mathematics and Natural Sciences, BRAC University, for allowing and encouraging us to complete our undergraduate thesis. Also, our heartfelt gratitude and appreciation go to **Nadia Sultana Deen**, Program Coordinator of the Microbiology program, Department of Mathematics and Natural Sciences, BRAC University, for all of the assistance, kindness, and guidance she showed us whenever we require it.

Our regards, gratitude, indebtedness, and appreciation go to our respected supervisor **Md Hasanuzzaman**, Senior Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University for his constant supervision, constructive criticism, expert guidance, enthusiastic encouragement to pursue new ideas, and never-ending inspiration throughout the entire period of our research work. We would also like to express gratitude toward **Akash Ahmed**, Senior Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University for his guidance and assistance.

Our sincere appreciation goes out to Laboratory Officers **Md. Mahmudul Hasan, Shamima Akhter Chowdhury**, and **Asma Binte Afzal** for their unwavering kindness and advice. Additionally, we would like to express our gratitude to our Research assistant **Nishat Tasnim Ananna** for their exceptional kindness and assistance. We would like to express our sincere

gratitude to you for guiding us through the writing of the report and occasionally offering suggestions regarding the setting of experimental designs, the interpretation of results, and subsequent directions for the entire project. With their warm assistance, it was possible to submit our report. In addition, we would like to express sincere and loving gratitude to our lab-mate **Sumaiya Morshed Aurpa**, **Riyan Mehjabin Ferdous**. and **Rezwana Afrin Adiba** for all their moral support and help throughout this challenging journey.

Additionally, we would like to express our appreciation to lab attendants **Ashik-E Khuda**, **Tanzila Alam**, and office attendant **Nadira Yeasmin** for their assistance during our eight months in the laboratory. Last, but not the least, we would like to express our gratitude to everyone who assisted us in the laboratory and worked alongside us.

Table of Contents:

List of Tables:

List of Figures:

List of Graphs:

List of Acronyms:

ABR	Antibiotic Resistance
AMR	Antimicrobial Resistance
AST	Antimicrobial Susceptibility Test
BL	Beta-lactamases
bp	Base pair
BPW	Buffer Peptone Water
CAI	Classical Acquired Infections
CAP	Community-acquired Pneumonia
cKp	Classical *Klebsiella pneumoniae*
CLSI	Clinical and Laboratory Standards Institute
COVID-19	CoronaVirus Disease 2019
DNA	Deoxyribose Nucleic Acid
DNCC	Dhaka North City Corporation
EDTA	EthylenediamineTetraacetic Acid
ESBL	Extended Spectrum Beta-lactamases
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GI	Gastrointestinal Tract
HAI	Healthcare-associated Infections

HAP	Hospital-acquired Pneumonia
hvKp	Hypervirulent *Klebsiella pneumoniae*
ICU	Intensive Care Unit
KPC	*Klebsiella pneumoniae* Carbapenemase
MCT	Micro Centrifuge Tube
MDR	Multi-drug Resistance
MHA	Mueller Hinton Agar
PCR	Polymerase Chain Reaction
SDG	Sustainable Development Goals
RNA	Ribonucleic Acid
rpm	Revolutions per Minute
RT	Reverse Transcriptase
TE	Tris-EDTA
TBE	Tris-borate-EDTA
UTI	Urinary Tract Infection
UV	Ultra Violet
WHO	World Health Organization

CHAPTER-1

INTRODUCTION

Klebsiella pneumoniae is a gram-negative opportunistic bacteria part of the ESKAPE group formed with the following microbial species: ***Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,*** and ***Enterobacter*** species that cause different types of infections like pneumonia, nosocomial infections, UTIs, etc. (Cordova-Espinoza et al., 2023). This microbe was documented as one of the top three pathogens of worldwide concern by the World Health Organization (WHO) in 2017 (Denissen et al., 2022). It has also been listed in the Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery, and Development of New Antibiotics.

Universal healthcare is a major key in achieving several sustainable development goals (SDGs). At present time, Antimicrobial resistance (AMR) is the main opposition in achieving the goals. Improper use of antimicrobials, specifically antibiotics on humans, animals, crops, and veterinary medicines causes resistance to the drugs. The resistance threatens the successful treatment of infections caused by bacteria. In 2017, WHO established a multi-criteria analysis where three priorities were ranked: **i) medium, ii) high, and iii) critical**. (Denissen et al., 2022) In priority 1 named ‘critical’, the Multidrug Resistance of gram-negative bacteria especially the ESKAPE group was listed. ***Klebsiella pneumoniae*** was listed for third-generation cephalosporin resistance (Denissen et al., 2022). AMR can occur due to three processes: **i) enhance or lower the efflux by enzymes that cause antibiotic modification, ii) alteration in antibiotic target sites, and iii) capability to break or change antibiotics.** (Ripon et al., 2023). It is estimated that globally around 700,000 (Otaigbe & Elikwu, 2023) deaths per year are caused due to AMR. AMR-related morbidity and mortality affect normal livelihood by a great margin.

Antibiotic resistance (ABR) in ***Enterobacteriaceae*** including ***Klebsiella pneumoniae*** is a vital health issue. It is one of the leading pathogens related to the emergence of antibiotic resistance. The bacteria is found in the natural flora of mammals' particularly in the intestinal tracts. Due to over usage of antibiotics rather than treating the infection, the drugs are giving the microbe more arsenal to develop into a serious disease. Alongside the pathogenicity of ***K. pneumoniae***, AMR is a major public health concern. The last line of antibiotics such as beta-lactams and carbapenems have been in use since the 1950s. The isolates that are resistant to these antibiotics are more or less resistant to all antimicrobial agents. This issue creates a high morbidity and mortality rate. In most studies, the mortality rate of ***K. pneumoniae*** ranges from 11% (Li et al., 2023) to 81% (Li et al., 2023). The best-defined mortality rate is within infectious diseases. Moreover, ESBL-producing and carbapenem-resistant isolates have a higher death rate compared to non-resistant bacteria. In one systematic review of 157 studies, the mortality rate stacked over 17% (Li et al., 2023) at the 7-day mark, 34% (Li et al., 2023) at the 90-day mark, and a total of 29% (Li et al., 2023) in hospital settings. More than 50% (Li et al., 2023) of the patients were admitted to the ICU.

In this study, the isolation of ***Klebsiella pneumoniae*** from hospital wastewater and their adjacent community tap water of Dhaka Metropolitan City was focused to find ESBL (Extended-spectrum Beta-lactamase) encoding and Carbapenem-resistant isolates. A 4-month (December 2022 to March 2023) long study was conducted in three different hospital areas and their surrounding community from where the isolates were selected primarily based on morphology in bacteriological media followed by genus-specific PCR and Antimicrobial Susceptibility Test (AST). After that, ESBL and Carbapenem-resistant gene findings were focused on the isolates.

The target of the study was to find the same type of AMR pattern of hospital wastewater isolates in the community tap water isolates to investigate the effect of hospital waste on antimicrobial resistance development. The reason for setting this target was due to similar studies found in developed countries whose results signify a big concern for the health sector in the modern age. On the other hand, Bangladesh is a developing country and the capital city Dhaka is overpopulated and the infrastructure of architecture is quite poor. So along with a global overview domestic surveillance data has to be obtained to take measures in the public health sector about the dynamic spread of antibiotic-resistant ***K. pneumoniae***.

This study is focused to highlight the potential hazard factors involving untreated hospital effluents which can and surely contaminate the surrounding community tap water. It is already established that ***Klebsiella pneumoniae*** shows resistance toward broad-spectrum antibiotics. The ability to resist multiple antibiotics has been gained due to the overuse of antibiotics on in-patients by clinicians in an empirical setting rather than pathogen-specific treatment. The patients' wastes are discarded in the sewage system where the microbes are being spread in the environment due to poor infrastructure of the hospital wastewater purifying treatment. Hospital-acquired pneumonia isolates are more likely to be resistant to antibiotics than community-acquired isolates due to untreated hospital effluents containing low doses of antimicrobials which can contribute to AMR in community tap water isolates. It is quite important to treat the wastewater according to provided guidelines so that the microbes from the untreated effluent are not transferred to community tap waters in bulk amounts and cause serious infections like nosocomial infections which will consequently put public health at great risk. Over time from the transfer of resistant ***K. pneumoniae*** in the community water, the classical

isolates can gain the resistant genes via mutation and selection along with genetic exchange which will ultimately arise a serious issue.

CHAPTER-2

LITERATURE REVIEW

2.1 - *Klebsiella pneumonia*

Klebsiella pneumoniae is one of the most common opportunistic microbial species responsible for pneumonia infections as well as nosocomial infections (Cordova-Espinoza et al., 2023). This bacterium is part of the ESKAPE group which is formed with the following microbial species: ***Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,*** and ***Enterobacter*** species (Cordova-Espinoza et al., 2023). This microbe belongs to the *Bacteria* domain, ***Proteobacteria*** phylum, ***Gammaproteobacteria*** class, ***Enterobacteriales*** order, ***Enterobacteriaceae*** family, and ***Klebsiella*** genus (Ashrust & Dawson, 2023). ***K. pneumoniae*** is a gram-negative bacteria that is fermentative and also encapsulated bacillus (Valenzuela-Valderrama et al., 2019) (Ashrust & Dawson, 2023). It is a non-motile bacterium meaning any means of movement is not possible (Valenzuela-Valderrama et al., 2019). This species of bacteria is mostly found in the environment including soil, surface water, and medical devices (Valenzuela-Valderrama et al., 2019).

In 1882, ***Klebsiella pneumoniae*** was first narrated by Carl Friedlander (Ashrust & Dawson, 2023). Friedlander isolated the bacterium from dead pneumonia patients' lungs and reported it as an encapsulated bacillus. Due to this, the bacterium was identified as Friedlanders' bacillus. After a few years, in 1886, the bacterium gained the name ***Klebsiella pneumoniae*** (Ashrust & Dawson, 2023).

2.2 - Diseases Caused by *K. pneumoniae*

K. pneumoniae can be found in many mammals' natural flora, especially the intestinal tract. It has been confirmed that the bacteria colonizes 39% (Wyres et al., 2020) of dogs (***Canis lupus***

familiaris) and 44% (Wyres et al., 2020) of dairy cattle (***Bos taurus***) intestines. In the human ***(Homo sapiens***) body, the bacteria is found in mucosal surfaces which include the gastrointestinal tract (GI) as well as the oropharynx (Wang et al., 2023). Although the effect of the colonization of the bacteria is benign yet the entry into the tissue can cause dangerous infections such as pneumonia, UTI (urinary tract infection), bacteremia, sepsis, meningitis, and pyogenic liver abscesses in the absence of biliary tract disease (Wang et al., 2023)(Paczosa & Mecsas, 2016) (Wyres et al., 2020). The bacterium ranks in the top three microbes to cause neonatal sepsis (Wyres et al., 2020). Moreover, ***K. pneumoniae*** is responsible for healthcare-associated infections (HAI) which are sometimes referred to as classical acquired ***K. pneumoniae*** infections (CAIs) (Wyres et al., 2020), for example, central line-associated bloodstream infections, catheter-associated urinary tract infections, wound infections, surgical site inflammation and infection, and ventilator-associated pneumonia (Cordova-Espinoza et al., 2023) (Wyres et al., 2020). The most vulnerable patients are from the age group of neonates and the elderly. In clinical cases, the bacterium is often isolated from the lungs of immunosuppressed patients in the intensive care unit(ICU) (Cordova-Espinoza et al., 2023)

Klebsiella pneumoniae can be introduced into the human body through two distinctive categories: community-acquired pneumonia (CAPs) and hospital-acquired pneumonia (HAPs) (Wang et al., 2023). Worldwide hypervirulent ***K. pneumoniae*** (hvKp) has emerged which is a virulent subtype (Wang et al., 2023). In contrast, the original strain is pointed out as classical *K. pneumoniae* (cKp) (Wang et al., 2023) (Paczosa & Mecsas, 2016). Usually, the hypervirulent strains cause more community-acquired infections and systemic infections in healthy individuals (Wang et al., 2023). Yet, these strains are limited to a geographical area, likely Taiwan and South

Asia (Paczosa & Mecsas, 2016). The hvKp strain can cause primary liver abscesses among patients who never had any liver disease (Paczosa & Mecsas, 2016). The infection can be started from the GI tract which led the bacterium to invade tissue. The liver abscesses can cause other secondary infections such as cellulitis, necrotizing fasciitis, myositis, endophthalmitis, and abscesses in other tissue sites (Wang et al., 2023) (Paczosa & Mecsas, 2016).

On the contrary, the classical strains cause primary infections typically nosocomial infections and UTIs which are worldwide phenomena. This also falls under the category of HAPs. A frequent cause of HAPs leads to a high rate of mortality due to multidrug resistance compared to CAPs as the patients are already treated with antibiotics and medications of antibiotic-resistant flora (Paczosa & Mecsas, 2016). Furthermore, CAPs cause potentially severe infections which progress to deteriorated health leading to hospitalization as well as high rates of morbidity and mortality (Paczosa & Mecsas, 2016). Most of the time, in Asia, people infected with CAPs of ***Klebsiella pneumoniae*** require mechanical ventilation. The representative symptoms of CAPs are acute pneumoniae, cough, fever, leukocytosis, and chest pain (Paczosa & Mecsas, 2016). Another type of symptom is ‘currant jelly sputum’ which is a thick blood-coated mucous (Paczosa & Mecsas, 2016). The cKp strains are also responsible for UTIs just behind Escherichia coli. Most of the time the infection arises from the colonization of the GI tract. Transfer of the bacterial strains into the bladder results in UTI. From the spread of the primary infection of the lungs and bladder, serious bacteremia can occur. This type of bacteremia can evolve into nosocomial bacteremia which has a high fatality rate (Paczosa & Mecsas, 2016).

2.3 - Epidemiology of *K. pneumoniae*

It has been thoroughly discussed that ***Klebsiella pneumoniae***, an opportunistic bacteria, is part of human microflora. In general, humans carry 5% to 38% (Paczosa & Mecsas, 2016) of the microbe in their stool and 1% to 6% (Paczosa & Mecsas, 2016) in their nasopharynx (Ashrust & Dawson, 2023). During the discussion of the diseases caused by ***K. pneumoniae***, it has been noticed that the main reservoir of the microbe is the GI tract and bladder. The rate of patients who carry HAPs is higher than the community-acquired ones. It can be estimated that around 77% of the bacteria can be seen in the stools of hospitalized patients (Ashrust & Dawson, 2023). Overall, worldwide 11.8% (Paczosa & Mecsas, 2016)of cases are of HAPs (Ashrust & Dawson, 2023). Of those, 8% to 12% (Ashrust & Dawson, 2023) are ventilated patients while only 7% (Ashrust & Dawson, 2023) are non-ventilated. It has also been noted in a study that in China, patients with severe alcoholism and septicemia are more prone to ***Klebsiella pneumoniae*** infection (Ashrust & Dawson, 2023). Otherwise, there is a high-risk factor for diabetic patients for pyogenic liver abscesses due to CAIs (Wyres et al., 2020).

In Western cultures like North America, Europe, and Australia, CAPs are very much lower compared to other countries, approximately 3% to 5% (Paczosa & Mecsas, 2016) (Ashrust & Dawson, 2023). While in developing countries like Africa and Asia, ***K. pneumoniae*** is only second to ***Streptococcus pneumoniae*** with a much higher rate of infection 15% (Paczosa & Mecsas, 2016). Various reports estimate that the CAP comprises 22% to 32% (Paczosa & Mecsas, 2016,) of ICU cases while the mortality rate dwindles between 45% to 72% (Paczosa & Mecsas, 2016). In another report, a clear assessment has presented that the mortality rate is 55% (Paczosa & Mecsas, 2016) among hospitalized patients who get infected due to CAPs. In

community-acquired UTI infection, the rate is 4.3% to 7% (Paczosa & Mecsas, 2016) while nosocomial infection is only about 2% to 6% (Paczosa & Mecsas, 2016). The prolonged UTI leading to bacteremia has a high rate of 50%.

A report from China shows a high ubiquity of hvKp ranging from 31% to 37.8% (Wang et al., 2023). Of all types of pneumonia, around 3% to 8% (Wang et al., 2023) have been induced by hvKp strain, and in 5 years the mortality rate increased from 40% to 60% (Wang et al., 2023).

2.4 - Antibiotics

Antibiotics or Antimicrobials are a type of antibacterial agent derived from the natural metabolites of microorganisms like Fungi and ***Actinomycetes***. This marvelous event was first observed by Sir Alexander Fleming in 1928 (Rahman et al., 2018). This inhibitory agent was named penicillin. Production of antibiotics has been categorized into **i)** semisynthetic, **ii)** modified natural products, and **iii)** chemically designed synthetic (Rahman et al., 2018). According to the target sites of the agents, there are five mechanisms of action for antibiotics: **i)** cell wall synthesis inhibition, **ii)** cytoplasmic membrane inhibition, **iii)** bacterial protein synthesis inhibition, **iv)** nucleic acid synthesis blocker, and **v)** folic acid synthesis blocker (Rahman et al., 2018).

2.5 - Beta-lactam Antibiotics

Among many classes of antibiotics, one is beta-lactam. This class of antibiotics is the most important in the category of cell wall synthesis inhibition (Rahman et al., 2018). This is a

bactericidal agent which specifically lyses the bacterial cell walls. Due to the chemical structure of the beta-lactam ring, there are six groups available: **i)** penicillins, **ii)** cephalosporins, **iii)** cephamycins, **iv)** carbapenems, **v)**monobactams, and **vi)** beta-lactamase inhibitors (Rahman et al., 2018,). The beta-lactam ring consists of a thiazolidine ring linked to the complex of three carbon atoms and one nitrogen atom (Rahman et al., 2018). After the occurrence of penicillin resistance, the next generation of antibiotics discovered was cephalosporin, leading to 2nd-generation - **i)** cefoxitin, **ii**) cefotetan, **iii)** cefmetazole, **iv)** cefaclor, **v)** cefpodoxime, **vi)** cefuroxime, 3rd-generation - **i)** cefixime, **ii)** cefodizime, **iii)** cefotaxime, **iv)** cefteram, **v)** ceftizoxime, and 4th-generation- **i)** cefepime, **ii)** ceflupreneme, **iii)** cefoselis, **iv)** cefozopran, **v)** cefpirome, **vi)** cefquinome (Rahman et al., 2018). In cephalosporin, the dihydro-thiazine and the beta-lactam ring are merged which is different in carbapenem, where the beta-lactam ring has a side chain of hydroxyethyl without any oxygen and sulfur atom in the bicycle nucleus (Rahman et al., 2018). On the other hand, monobactams do not have any additional rings.

When antibiotics are unable to inhibit the growth of the pathogen microbe, the term drug tolerance or drug failure comes up. This event occurs when the microbes become resistant to the effect of the antibiotics. Worldwide, around 50% (Rahman et al., 2018) of used antibiotics are from the beta-lactam group which creates an unfortunate situation of increased resistance towards an efficient antibiotic class. Currently, four major ways have been known for the bacteria to resist beta-lactams. Among the four, the most common and used mechanism for ***Enterobacteriaceae*** like ***K. pneumoniae*** is the production of beta-lactamases (BLs), a type of enzyme that hydrolyses the beta-lactam ring and inactivates the mechanism of the antibiotics (Rahman et al., 2018).

2.5.a - Beta-lactamase Enzyme

In gram-negative microbes, the beta-lactamase enzyme is the most frequently used resistance mechanism. The mechanism either works through plasmid or is expressed chromosomally (Rahman et al., 2018).

There are two classification systems available for the enzyme: **i) Ambler Molecular Classification** - it is based on the conserved motifs and protein sequences where detailed categorization occurs through **classes A, B, C, and D** enzymes. Though these enzymes serine is utilized for beta-lactam hydrolysis also the metalloenzymes of class B require divalent zinc ions to substrate the hydrolysis process. **ii) Bush, Jacoby, and Medeiros Functional Classification** - in this case, a grouping of different beta-lactamases according to their substrate and inhibitor profiles occur. There are three groups - **a) group 1 (class C)** - cephalosporinases, **b) group 2** (classes A and D) - broad-spectrum, inhibitor-resistant, extended-spectrum beta-lactamases, and serine carbapenemases, and **c) group 3 (class B)** - metallo beta-lactamases (Rahman et al., 2018).

2.5.b - Extended Spectrum Beta-lactamase (ESBL)

ESBL or extended-spectrum-beta-lactamases is part of the beta-lactamase enzyme. The ESBLs are plasmid born and can hydrolyze the 3rd and 4th generation cephalosporin named oxyimino-cephalosporin and monobactams but not carbapenems including meropenem and imipenem (Rahman et al., 2018). Originally, the ESBLs are derived from a narrow-spectrum parent ESBL enzyme and they can inactivate the penicillins, aztreonam, and broad-spectrum cephalosporins yet excluding carbapenems (Rahman et al., 2018). The mechanism works by

hydrolytic activity and inhibition of beta-lactamase inhibitors named clavulanic acid. The older ESBLs are derivatives of TEM-1, TEM-2, and SHV (Rahman et al., 2018). Among ***Klebsiella pneumoniae***, extended-spectrum beta-lactamases-producing strains are important pathogens responsible for nosocomial infections which can extend to serious bacteremia and pneumonia (Filis et al., 2021). The multidrug resistance (MDR) state of ***K. pneumoniae*** has led to the emergence of incurable diseases.

ESBL has been divided into three main groups:

i) **$ESBL_A$ (Class A)** - the most frequently found ESBLs, CTX-M, SHV, and TEM enzymes. The enzymes are horizontally transferable and inactivated by clavulanic acid.

ii) **$ESBL_M$ (miscellaneous ESBL)** - there are two sections: a) $ESBL_{M\text{-}C}$ (class C, plasmid-mediated AmpC) and $ESBL_{M\text{-}D}$ (class D)

iii) **$ESBL_{CARBA}$(ESBLs that degrade carbapenems)** - there are three subsections: a) $ESBL_{CARBA\text{-}A}$, b) $ESBL_{CARBA\text{-}B}$, and c) $ESBL_{CARBA\text{-}D}$.

2.5.c - Types of ESBLs

In a variety of pathogens, many types of ESBLs have been found. Gram-negative bacterial species such as ***E. coli*** and ***K. pneumoniae*** adopted two strategies of evolution. The strategies are - **i)** the assortment of mutants with extended substrate specificity from the already prevalent TEM and SHV types of beta-lactams. and **ii)** uptake and capturing of novel broad-spectrum-beta-lactamases genes from the naturally existing metagenome, coding for enzymes naturally endowed with ESBL activity (Rahman et al., 2018).

2.5.c.I. CTX-M

CTX-M enzyme has been recently discovered but it has been reported the most in terms of resistance. The enzyme has been titled after the extended activity against cefotaxime contrasting ceftazidime (Rahman et al., 2018). In terms of predominance abundance, CTX-M is far more effective and actively toppled other ESBL types such as TEM enzymes. Recently, the enzyme has been reported with well-defined amino acid sequences and functional characteristics (Rahman et al., 2018). Oftentimes the expression of the enzyme is correlated with the expressions of other enzymes leading to a critical reduction of treatment ability. CTX-M-type of enzymes are inherited by lateral gene transfer from the ***Kluyvera* sp.** (Rahman et al., 2018). There are six groups of this enzyme: **i) CTX-M-1, ii) CTX-M-2, iii) CTX-M-8, iv) CTX-M-9, v) CTX-M-25, and vi) KLUC** (Rahman et al., 2018). There is more than 94% (Rahman et al., 2018) amino acid similarity among the members and around 90% (Rahman et al., 2018) similarity overall have been observed. Furthermore, four variants express a hybrid structure such as CTX-M-45 which was formerly named Toho-2, which is a hybrid of CTX-M-14 (Rahman et al., 2018). It has an unknown origin of protein structure. Then, CTX-M-64, CTX-M-123, and CTX-M-132 are hybrids of CTX-M-15 (Rahman et al., 2018). These hybrids have different segments of CTX-M-14 present in them. The original variants of the enzyme are biologically different from the hybrids. Globally, CTX-M-15 and CTX-M-14 are the most commonly detected followed by CTX-M-2, CTX-M-3, and CTX-M-1 (Rahman et al., 2018).

2.5.c.II. TEM

Among the bacterial species, gram-negative bacteria mostly encode for the TEM enzyme (Rahman et al., 2018). Around 90% (Rahman et al., 2018) of resistance against ampicillin is due

to TEM enzymes. This enzyme is plasmid-mediated which is evolved from classic TEM mutations. Classic TEM consists of TEM-1 and TEM-2 genes (Rahman et al., 2018). There are single or multiple amino acid substitutes available in the active sites (Rahman et al., 2018). TEM-1 hydrolyzes penicillin and the 1st generation cephalosporin (cephaloridine). On the other hand, TEM-2 evolved from the TEM-1 enzyme due to mutations in the amino acid sequences (Rahman et al., 2018). They each have similar hydrolytic points but different isoelectric points, thus not considered ESBL. In 1987, a new plasmid-mediated beta-lactamase named CTX-1 was isolated from ***K. pneumoniae*** (Rahman et al., 2018). This enzyme is now called TEM-3 (Rahman et al., 2018). Accordingly, other TEM enzymes have been discovered. TEM-12 was detected in ***Klebsiella*** sp. which was considered the 1st TEM-type ESBL (Rahman et al., 2018).

2.5.c.III. SHV

In ***Klebsiella*** sp. Mostly ***K. pneumoniae***, plasmid-mediated SHV enzymes are found frequently. This enzyme distinguishes the sulfhydryl variables according to the belief that SHV is inhibited by chloromercuribenzoate due to its' relation with the substrate (Rahman et al., 2018). The 1st SHV-ESBL type detected was SHV-2 in ***Klebsiella ozaenae*** in 1983 from Germany (Rahman et al., 2018). Unlike previously seen TEM and CTX-M, SHV has comparatively fewer variants. Unlike the vast category of CTX-M, the substitution of amino acids is restricted to a narrower region.

2.5.c.IV. OXA

In ESBLs, the OXA-type enzymes are exceptionally increasing. This enzyme diverges from the SHV and TEM enzymes and fits perfectly into molecular class D and functional group 2d (Rahman et al., 2018). OXA enzyme exhibits oxacillin-hydrolyzing capabilities (Rahman et al., 2018). The enzyme is expressed in ***Pseudomonas aeruginosa***, unlike other ESBLs which are prevalent mostly in the ***Enterobacteriaceae*** family (Rahman et al., 2018). It shows resistance to cephalothin and ampicillin due to the high hydrolytic activity shown against oxacillin and cloxacillin (Rahman et al., 2018). OXA enzymes cannot diffuse the newer generation of cephalosporins so they are not regarded as ESBLs. There are OXA-ESBLs such as **OXA-11, OXA-14, OXA-16, OXA-17, OXA-19, OXA-15,OXA -18, OXA-28, OXA-31, OXA-32, OXA-35, and OXA-45** (Rahman et al., 2018).

2.5.d - Epidemiology ESBL Containing *K. pneumoniae*

It cannot be disregarded that the establishment of ESBLs in clinical practices in the 1980s prevented fatal infections and diseases caused by pathogens mainly of the ***Enterobacteriaceae*** family (Rahman et al., 2018). However, this resulted in the colossal use of cephalosporins which enabled the emergence of new ESBL variants. The new variants are progressively spreading in environment and clinical settings. In the epidemiology of ESBLs, quite several factors make it complex such as **i) geographical area, ii) country of origin, iii) hospitals, iv)community, v) host, vi) reservoirs, vii) mobile resistant element, viii) spread from the environment to water to animals, and ix) animals to human transmission** (Rahman et al., 2018). From the late 1990s to the early 2000s, there was an increase in the distribution of ESBLs in the pathogens especially ***E. coli*** and ***Klebsiella*** sp. (Rahman et al., 2018). This spike became a vital threat in

nosocomial infections. Members of TEM and SHV enzymes were prevalent in gram-negative bacteria like ***Klebsiella pneumoniae*** which caused the spread of infection in hospital settings (Rahman et al., 2018). In Sweden, ***K. pneumoniae*** having CTX-M-15 enzyme were reported which resulted in a nosocomial infection among neonates (Rahman et al., 2018). Moreover, in the late 90s, SHV and TEM were originally affiliated with ICU patients' nosocomial infection (Rahman et al., 2018). Also, the enzymes were more prevalent in ***K. pneumoniae***. In 2011 from Italy, a novel SHV-12 in ***K. pneumoniae*** have been reported (Rahman et al., 2018). Notably, the epidemiology of CTX-M compared to SHV and TEM is complex due to the fecal widespread route. Most of the infections related to CTX-M were reported from CAI. An increase in the enzyme in ***Klebsiella*** sp. is observed, at 28% (Rahman et al., 2018)in Bulgaria, 16% (Rahman et al., 2018) in Cyprus, and 12% (Rahman et al., 2018) in Romania. In Middle East countries, a random survey in 2008 concluded that 27% (Rahman et al., 2018) of ***K. pneumoniae*** strains carrying SHV-12 and TEM-1. In Asia, the prevalence of ESBLs is 68% (Rahman et al., 2018) in India, 52% (Rahman et al., 2018) in Pakistan, and 30% (Rahman et al., 2018) in China.

2.6 - Carbapenemase Activity of *K. pneumonia*

A global study has shown that around 20% (Pitout et al., 2015) to 80% (Pitout et al., 2015) of ***Klebsiella pneumoniae*** were resistant to first-class antibiotics like cephalosporins, fluoroquinolones, and aminoglycosides back in the 2000s (Pitout et al., 2015). The emerging resistance of carbapenems, which is the last-line antibiotic treatment plan for infections especially caused by ***K. pneumoniae,*** is a massive concern for the current health sector (Pitout et al., 2015). In some countries hospital-acquired infections of ***K. pneumoniae*** are difficult to treat for half of the patients due to carbapenem resistance.

The ability to resist carbapenems is caused by the production of beta-lactamases which simultaneously produce weak carbapenemase activity (Pitout et al., 2015). Apart from beta-lactamase activity, carbapenemases work without any additional permeable effect. These enzymes are part of the Ambler molecular class A, B, or D (Pitout et al., 2015). Class A carbapenemase has been uniquely accounted for in ***K. pneumoniae***. More than 20 variants of ***Klebsiella pneumoniae*** Carbapenemases (KPC) have been distinguished till today (Pitout et al., 2015). These enzymes show resistance to penicillins, cephalosporins, cephamycins, carbapenems, and monobactams (Pitout et al., 2015). Moreover, they are inhibited by boronic acid, avibactam, clavulanic acid, and tazobactam (Pitout et al., 2015). OXA-48 is the only class D carbapenem that hydrolyzes beta-lactamase usually found in ***K. pneumoniae*** isolates (Pitout et al., 2015). There are many derivatives of OXA-48 available such as OXA-181, OXA-204, and OXA-232 (Pitout et al., 2015). OXA-48 can hydrolyze narrow-spectrum beta-lactams like penicillin and weakly hydrolyzes carbapenems. Yet, no action towards broad-spectrum cephalosporins. Furthermore, carbapenems vary according to isolates and host permeability background. Carbapenemases have different hydrolytic activities which are more efficient than the OXA-48 enzyme. Nonetheless, high performance of resistance requires additional permeable deficiency despite the type of carbapenems that are being produced. In Addition to OXA and KPC, there are NDM, VIM, and IMP enzymes available in the carbapenemase category (Pitout et al., 2015).

2.6.a-Epidemiology of Carbapenemase Containing *K. pneumoniae*

In the late 1990s, KPC-1 containing ***K. pneumoniae*** isolates was reported in North Carolina (Pitout et al., 2015). KPC-2 and 3 have been found in many ***K. pneumoniae*** isolates. KPC enzyme-producing bacteria are regarded to cause endemics such as nosocomial infections in certain regions of the world, commonly in the northeastern United States, Colombia, Puerto Rico, Greece, Israel, Italy, and China (Pitout et al., 2015). There was a successful spread of ***K. pneumoniae*** containing bla_{KPC} back in the 1990s in the United States. In the same decade, ***K. pneumoniae*** was found in Greece containing bla_{VIM} (Pitout et al., 2015). OXA-48 was reported in a Turkish multi-drug-resistant (MDR) ***K. pneumoniae*** isolate found in Paris, France (Pitout et al., 2015). ***K. pneumoniae*** that produces OXA-48 causes endemic in mainly Turkey and other North African and European countries like Morocco, Tunisia, Spain, and Belgium. OXA-181, the most common derivative of OXA-48 is usually found in the Indian subcontinent. The other derivatives can be identified in North Africa, Australia, and New Zealand (Pitout et al., 2015). There are NDM, VIM, and IMP-type enzymes where NDM is found globally. Contrastingly, the IMP enzyme is mostly found in China, Japan, and Australia. While VIM is found in Italy and Greece (Pitout et al., 2015).

CHAPTER-3

METHODOLOGIES

3.1 Sample Collection Spot:

For the research findings 3 hospitals and their community area of Dhaka Metropolitan area more specifically Dhaka North City Corporation were chosen. The study was conducted from December 2022 to March 2023. The Hospitals from where the samples were collected were: ***Dhaka Shishu Hospital***, ***Dhaka North City Corporation(DNCC) COVID-19 Dedicated Hospital***, and ***National Cancer Hospital, Dhaka***. Among 3 of them ***DNCC COVID-19 Dedicated Hospita***l and ***National Cancer Hospital, Dhaka*** is situated in Mohakhali, Dhaka, Bangladesh. And ***Dhaka Shishu Hospital*** is situated in Shyamoli, Dhaka, Bangladesh. The community spot was marked around 200 meters from the hospital area and it was the same for 3 of the hospitals. The community locations were spotted around 300 meters by measuring the distances from the hospital wastewater location.

In each month samples were collected from three of the hospitals along with their adjacent community tap water. highlighting the potential risk that untreated hospital effluents dripped into the environment might lead to major disease outbreaks. Additionally, the targeted regions are frequently overcrowded with large numbers of people, namely patients and their attendants. Considering these reasons it can be said that these locations were suitable for carrying out this research.

Figure-1: Sample collection spots of the study which were inside Dhaka City

3.2 Sample Collection Process:

In the study, water is the source that was used to identify the designated organism ***Klebsiella pneumoniae***. For sampling, Hospital drainage water and hospital-adjacent Community tap water were collected. To collect the sample, autoclaved 500mL bottles for community water and a 45mL autoclaved Falcon for Hospital drainage water were used. On the sample collection day, those bottles and falcons were kept inside a carrier along with the ice bag to keep the temperature low. To avoid cross-contamination and collectors safety 1 pair of gloves, 1 zip lock bag, and masks were kept also. To collect the hospital drainage water a 45mL falcon tube was dipped inside the drainage and as it filled up the falcon was locked with a cap. After collecting the hospital drainage sample we kept the falcon inside a zip lock bag and kept it inside the carrier. After collecting the hospital drainage sample, the community water sample was collected in 500mL bottles which were filled with tap water and kept in the carrier containing sterile ice

packs. After collecting all the samples both hospital and community were named with a UNIQUE name containing the name of the month and hospital. Then kept the samples inside the carrier unopened till the sample processing. As soon as the sample collection was done the used gloves were kept inside a ziplock bag, and hands were cleaned with water following sanitation with 70% ethanol.

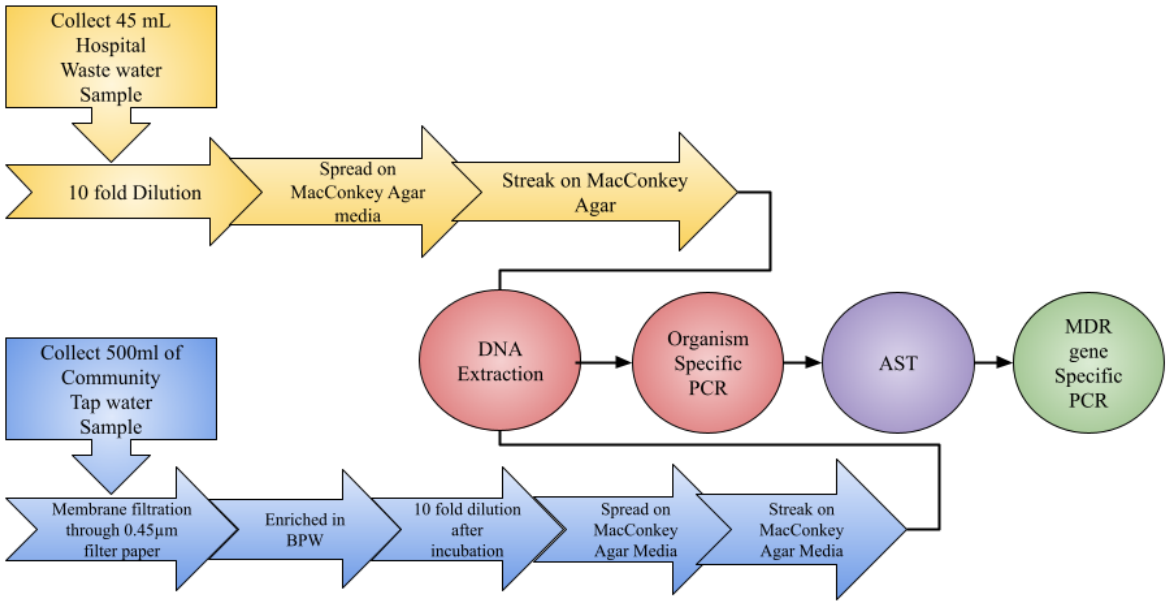


Figure-2: Workflow for Hospital and Community sample

3.3 Sample Processing:

Sample processing is a crucial part of the correct isolation and identification of the targeted organism. After collecting the sample they were kept in the carrier until the sample processing started. The sample was processed within 4 hours of the sample collection. There are various steps of sample processing and the processing procedures difference between the Hospital drainage water sample and community tap water samples.

3.3.a Filtration:

3.3.a.I. Community Sample:

For filtration "MEMBRANE FILTRATION METHOD" has been followed to filter the Community water samples. In the Membrane-Filtration Method microorganisms were removed from the apparatuses, especially from the foam used in the filtration. All the filtration apparatus of Membrane filtration were used by autoclaving them for purification and to stop cross-contamination. A 0.22-micrometer filter paper was used to trap the microorganisms present while filtrating the hospital water samples and each time separate filtration apparatuses were used to filter the water samples. Filtration techniques were:

- ➔ At first, the holding apparatus was placed and a foam to place the filter paper was placed as well.
- ➔ Then, a 0.22 micrometer-sized filter paper was placed on top of the soam of the filtration apparatus by sterilizing forceps which was sterilized by flaming it in the burner.
- ➔ Then the water-holding apparatus was on top of the filter paper and both the apparatus were attached through a clamp.
- ➔ After that, 100mL water was poured into the holding apparatus, and the machine was started to filter the water.
- ➔ As soon as the whole water passed through the filter, the machine stopped.
- ➔ After stopping the machine, the clamp and the holding apparatus were removed. Then the filter paper was removed from the foam very carefully through a sterilized forcape.

- ➔ After removing the filter paper, it was inoculated into the freshly autoclaved Buffer Peptone Water (BPW) contained in falcons.
- ➔ In the end, the falcons were kept in the shaker incubator for the incubation period which was 16-18 hours for the proper growth of the microorganisms.

3.3.b. Dilution:

3.3.b.I. Hospital Sample:

The hospital sample was the direct drainage water sample. To isolate the desired organism ***Klebsiella pneumoniae*** it needs to be diluted for easy isolation. For dilution, 9 mL autoclaved physiological saline (0.9%) was used.

- ➔ At first, saline 0.9% was made, poured 9mL saline on each test tube, and autoclaved.
- ➔ Then, a 100 microlitre hospital sample was taken from the sample containing falcon with a micropipette and put into the saline labeled as 10^{-1}.
- ➔ After that 10 fold dilution was done and each time the test tubes were vortexed for well-mixture.
- ➔ As soon as the dilution ended, the spread was done on selective media.

3.3.b.II. Community Sample:

As soon as the incubation period ended the turbidity of the BPW was checked. If turbidity of the BPW was observed, dilution was done from the BPW as same as the Hospital water dilution procedure. A 10-fold dilution was done for the community sample as well.

3.4 Isolation and Identification of the Targeted organism on Selective Media:

The targeted organism was ***Klebsiella pneumoniae*** and for the isolation of ***Klebsiella pneumoniae*** from the hospital wastewater sample and community water sample different selective media were used. As ***K. pneumoniae*** is a gram-negative organism MacConkey Agar media was used which distinguishes between gram-positive and gram-negative bacteria because MacConkey Agar media allows the growth of only Gram-negative bacteria. As ***K. pneumoniae*** is a gram-negative organism, MacConkey Agar was a suitable medium for the isolation of hospital wastewater and community water samples.

3.4.a Spread:

For the hospital wastewater sample, 100µl of the diluted sample of dilution factor 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} , and direct wastewater samples were taken for spreading on the MacConkey Agar. Hospital wastewater samples were processed within 4-6 hours of the sample collection timing. After spreading, plates were incubated at 37°C for 18-24 hours.

For community water samples, 100µl of diluted samples of the dilution factor 10^{-2}, 10^{-3}, and 10^{-4} were taken for spreading on the MacConkey Agar. After spreading, plates were incubated at 37°C for 18-24 hours.

3.4.b Streak:

As soon as the incubation period ended the spread plates were observed to identify the ***K. pneumoniae***. We used to look for Mucoid-shaped pink distinct colonies on the MacConkey Agar

plates which were marked on the assumption to isolate them as ***K. pneumoniae.*** The marked colonies were picked for streaking on the MacConkey agar plates to isolate single colonies. After streaking on the MacConkey agar plates they were incubated at 37°C for 18-24 hours. After this incubation period, the isolated single colonies were taken to streaked on the Nutrient agar plates and incubated at 37°C for 18-24 hours for the isolation of pure culture for further proceedings. These processes are similar for both hospital and community samples.

3.5 Molecular Analysis:

3.5.a DNA extraction:

After isolating the suspected target organism, to confirm it as ***K. pneumoniae*** DNA was extracted for further proceedings. A process to purify DNA by using physical and/or chemical methods from a sample that separates DNA from cell membranes, proteins, and other cellular components is known as DNA Extraction. Friedrich Miescher in 1869 did DNA isolation for the first time. (Gupta, 2019). There are various techniques for extracting DNA in both physical and chemical methods. For our findings, we used the Boiling method to extract DNA because it is the easiest way and less time-consuming method. It also needs fewer reagents to extract the DNA. An extraction buffer and some materials were needed to perform the purification of DNA. For DNA extraction 150µL of 1X TE buffer was taken in a micro centrifuge tube (MCT). Then, a loop full of organisms was taken and put inside the TE buffer. After that microcentrifuge tube was vortexed for a short time for homogenization. Then, it was heated through a dry heater at 100°C for 15 minutes. After completion, the heating microcentrifuge tubes were centrifuged for 5 minutes at 13,000 r.p.m. Then, the supernatant was collected on a separate centrifuge tube and stored at -20°C for further analysis.

3.5.b Conventional Polymerase Chain Reaction (PCR) and Gel Electrophoresis:

Polymerase Chain Reaction or PCR is a technique to make numerous copies of the DNA or RNA by amplifying them. PCR is used for various identification like paternity checks, identification of bacteria or viruses, and many more. Also, there are different kinds of PCR techniques available, like: conventional, Reverse Transcriptase(RT), Qualitative, Quantitative, and others. In our study, we use the Conventional PCR technique to amplify our bacterial DNA because in our laboratory that was the easiest and most available one to operate. To identify the targeted organism ***K. pneumoniae***, in each PCR mixture tube contains 6.5µl Mastermix(all the components), 2.5µl Nuclease Free water, 1µl of ***K. pneumoniae*** specific primers both Reverse(KP Pr1-R) and Forward(KP Pf-F) (KP Pf-F: 5′-ATT TGA AGA GGT TGC AAA CGA T-3′; KP Pr1-R:5′-TTC ACT CTG AAG TTT TCT TGT GTT C-3′)(Diaz et al., 2013) and 2µl of previously extracted DNA of the sample isolates. After mixing all the elements, PCR tubes were kept on the PCR Machine. Then the PCR was done for 35 cycles on the following conditions: starting from Initialization at 94°C for 10 mins, Denaturation at 94°C for 40 secs, Annealing at 54°C for 45 secs, Extension at 72°C for 45 secs and ends on Final Extension at 72°C for 10 mins(Diaz et al., 2013). After completion of the PCR, Agarose Gel electrophoresis was done. Agarose Gel Electrophoresis is a method to separate DNA fragments that vary in size of the DNA fragments. In our study, we use Horizontal Agarose Gel Electrophoresis to visualize the DNA amplified in the PCR technique. The amplified PCR products were run in a 1.5% agarose gel submerged in 1X TBE running buffer at 500 volt in an electrophoretic chamber for 50 minutes. Agarose gels were stained with Ethidium Bromide, mostly 4µl of this was used in 100ml of agarose gel for the clear visualization of the DNA bands of ***K. pneumoniae***. On each well of the gel, 4µl or

6µl(depending on the well size) of the PCR products were inoculated. Previously characterized as an isolate of ***K.pneumoniae*** from Dhaka Shishu Hospital, Dhaka was used as a positive control and 100 bp DNA Ladder was used to confirm the band size. (Cruz-Córdova et al., 2014)

3.5.c Phenotypic Analysis by Disk Diffusion Antibiotic Susceptibility Testing:

To study the phenotypical scenario of the PCR Confirmed isolates one of the most used techniques Antibiotic Susceptibility Testing was done. Antimicrobial susceptibility testing (AST) and the detection of the target microorganisms in the laboratory both yield crucial phenotypic information regarding an organism's morphological characteristics and antibiotic resistance(Coorevits et al., 2015). In our study, Disk Diffusion Antibiotic Susceptibility Testing was done on Muller-Hinton Agar(Main name) to identify if the isolates were Extended Spectrum β Lactamase (ESBL) and Carbapenem producing ***K. pneumoniae***. To identify those isolates following 11 antibiotics were used: Gentamicin from Aminoglycoside Class, Amikacin and Imipenem from Carbapenam Class, Cefixime and Ceftriaxone from 3rd Generation Cephalosporin class, Cefepime from 4th Generation Cephalosporin class, Norfloxacin, Erythromycin, Amoxicillin+Clavulanic Acid, Doxycycline from Marcolids class, Aztreonam from Monobactam class. AST. At first, the PCR confirmed ***K. pneumoniae*** isolates were cultured on Nutrient agar. Then from the pure culture, the isolates were taken and inoculated onto the 5 ml of 0.9% saline solution. Then it was vortexed and compared with the McFarland solution of standard 0.5. After that, freshly autoclaved cotton swab sticks were dipped into the saline solution and then lawned on the MHA media evenly. The lawn needs to be evenly spread otherwise the diffusions will not be done properly. After the lawn, the antibiotic discs were

placed on the lawned plates and then incubated for 18-24 hours. After completion of the incubation period, the zone of inhibition of the plates was measured to identify the Sensitivity or Resistance pattern. According to the CLSI and EUCAST standards, zones of inhibition have been defined as Susceptible (S), Intermediate (I), or Resistant (R). Various steps were followed to perform the Disk Diffusion. (Coorevits et al., 2015)

Name of the Antibiotics	Sensitive	Intermediate	Resistant
Gentamicin	≥ 17mm	-	≤ 17
Amikacin	≥ 18mm	15-17mm	≤ 15mm
Imipenem	≥ 23mm	20-22 mm	≤ 19 mm
Cefixime	≥ 19mm	16-18 mm	≤ 15 mm
Ceftriaxone	≥ 23 mm	20-22 mm	≤ 19 mm
Cefepime	≥ 25 mm	19-24 mm	≤ 18mm
Norfloxacin	≥ 17 mm	13-16 mm	≤ 12 mm
Erythromycin	≥ 23mm	14-22 mm	≤ 13mm
Amoxicillin+Clavulanic Acid	≥ 18mm	14-17 mm	≤ 13 mm
Doxycycline	≥ 14 mm	11-13 mm	≤ 10mm

Aztreonam	≥ 21 mm	18-20 mm	≤ 17mm

Table 1- Sensitive, Intermediate, and Resistant measurement of *Enterobacteriaceae* in mm

3.5.d Genotypic Analysis by Multidrug Resistance (MDR) Gene Detection:

In the study, the main motto was to identify and analyze whether the identified ***Klebsiella pneumoniae*** contains any MDR gene. After visualizing and analyzing the AST data MDR Gene detection was performed. For the detection purpose, Gene detection-based PCR was performed only for those isolates who had more resistance patterns than other isolates. To identify the targeted gene on organism ***K. pneumoniae***, each PCR mixture tube contains 6.5/6.0µl Mastermix which includes Dna polymerase (taq) , dNTPs , Mgcl2 , 2X buffer (EmeraldAmp® GT PCR Master Mix), 2.5/3.5µl Nuclease Free water, 1/0/75µl of gene-specific primers both Reverse and Forward and 2µl of previously extracted DNA of the sample isolates. After mixing all the components a short spin was given for mixing and then put onto the PCR machine. To analyze the presence of the MDR gene in the confirmed isolates following gene-specific primers had been used to perform the Gene specific PCR:

Gene Name	**Primer sequence**	**PCR Conditions**	**Base Pair**	**Reference**
$bla_{\text{NDM-1}}$	F-5′-GGTTTGGCGATCTGG TTTTC-3′ R-3′-CGGAATGGCTCATCA	**Initial Denaturation-** 94°C for 5 mins **Denaturation-** 94°C for 30sec	621	(Agarwal et al., 2018)

	CGATC-5′	**Annealing-** 58°C for 30 sec **Extension-** 72°C for 30 sec **Final Extension-** 72°C for 7 mins		
bla$_{CTX-M}$	F-5′ACGCTGTTGTTAGGAA GTG-3′ R-3′TTGAGGCTGGGTGAA GT-5′	**Initial Denaturation-** 94°C for 5 mins **Denaturation-** 94°C for 30sec **Annealing-** 58°C for 30 sec **Extension-** 72°C for 30 sec **Final Extension-** 72°C for 7 mins	759	(Zhang et al., 2021)
SHV	F-5′-TACCATGAGCGATAA CAGCG-3′ R-3′-GATTTGCTGATTTCG CTCGG-5′	**Initial Denaturation-** 94°C for 5 mins **Denaturation-** 94°C for 30sec **Annealing-** 58°C for 30 sec **Extension-** 72°C for 30 sec **Final Extension-** 72°C for 7 mins	450	(Doosti et al., 2015)
bla$_{KPC}$	F-5′-CATTCAAGGGCTTTC TTGCTGC-3′ R-3′-ACGACGGCATAGTCA	**Initial Denaturation-** 95°C for 5 mins **Denaturation-** 95°C for 50sec	498	(Mahmoud et al., 2020)

	TTTGC-5′	**Annealing-** 57°C for 30 sec **Extension-** 72°C for 40 sec **Final Extension-** 72°C for 10 mins		
bla$_{VIM}$	F-5′-GGTGTTTGGTCGCAT ATCGCAA -3′ R-3′-ATTCAGCCAGATCGG CATCGGC -3′	**Initial Denaturation-** 95°C for 5 mins **Denaturation-** 95°C for 45 sec **Annealing-** 60°C for 45 sec **Extension-** 72°C for 1 min **Final Extension-** 72°C for 8 mins	501	
bla$_{TEM}$	F-5'-AAAATTCTTGAAGAC G-3' R-3'-TTACCAATGCTTAAT CA-5'	**Initial Denaturation-** 95°C for 3 mins **Denaturation-** 95°C for 30sec **Annealing-** 51°C for 30 sec **Extension-** 72°C for 30 sec **Final Extension-** 78°C for 7 mins	1100	(Zhang et al., 2019)
bla$_{IMP-1}$	F-5′-GAAGGCGTTTATGTTCAT AC-3′	**Initial Denaturation-** 95°C for 5 mins **Denaturation-** 95°C for 50sec	587	(Khosravi & Mihani, 2008)

	R-3′- GTATGTTTCAAGAGTGAT GC-5′	**Annealing-** 57°C for 30 sec **Extension-** 72°C for 40 sec **Final Extension-** 72°C for 10 mins		
$bla_{\text{OXA-48}}$	F-5′-GCTTGATCGCCCTCG ATT-3′ R-3′-GATTTGCTCCGTGGC CGAAA-5′	**Initial Denaturation-** 94°C for 5 mins **Denaturation-** 94°C for 30sec **Annealing-** 50°C for 30 sec **Extension-** 72°C for 30 sec **Final Extension-** 72°C for 7 mins	281	(Gurung et al., 2020)

Table-2: Information of MDR Gene for PCR analysis.

CHAPTER-4

RESULT INTERPRETATIONS

&

OBSERVATIONS

4.1 Isolation of *Klebsiella pneumoniae*

In the study period from December 2022 to March 2023 a total of 52 samples had been collected from hospital wastewater and community tap water. Among the 52 samples 12 samples were from hospital wastewater and the remaining 40 samples were from hospital adjacent community tap water. On the primary basis from the 52 samples, a total of 154 isolates were counted as suspected ***K. pneumoniae*** by observing their colony morphology on selective MacConkey Agar media. From those 154 isolates, 79 isolates were from hospital wastewater and the remaining 75 isolates were from community tap water. To identify whether these isolates were ***K. pneumoniae*** or not species specific PCR was done routinely.

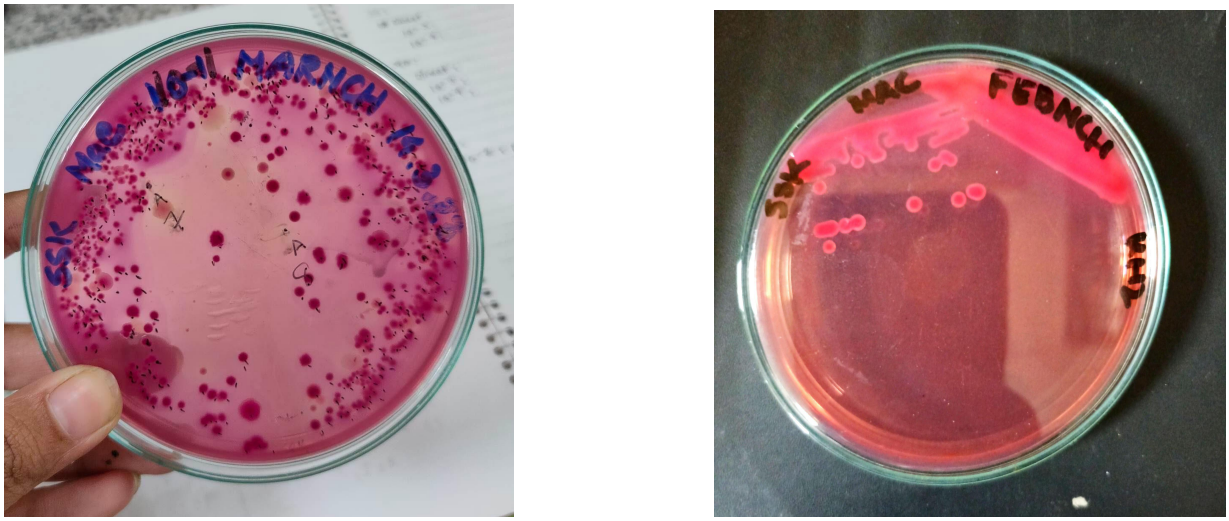
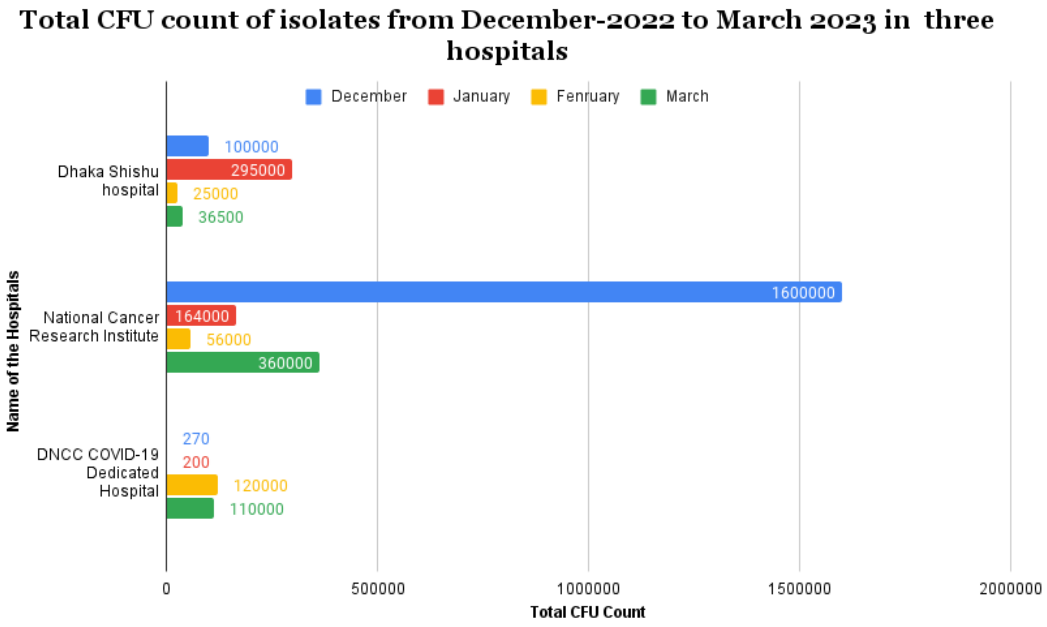


Figure-3 : Pink Mucoid shaped colonies on MacConkey Agar media

According to Total CFU counts of the hospital samples, the highest number of colony formation was seen in December-2022 from National Cancer Research Institute and Hospital. But in the same month in Dhaka shishu hospital and FNCC COVID-19 Dedicated hospital Total CFU Counts decreased drastically. In January from Dhaka Shishu hospital the highest number of total CFU count was the highest but for the other two hospitals the number was the lowest. In February-2023, from DNCC COVID-19 Hospital the highest number of Total CFU counts was counted and other two were lower than this. In March-2023, Total CFU count number was the highest for the National Cancer Research Institute and Hospital and lowest for Dhaka Shishu hospital. After analyzing the whole data, it was observed that the highest number of colonies counted for National Cancer Research Institute and Hospital and the lowest was DNCC COVID-19 Dedicated Hospital.



Graph-1: Total CFU count of isolates from December-2022 to March 2023 in three hospitals

4.2 Identification of Klebsiella pneumoniae by PCR Assay and Gel electrophoresis

After the completion of PCR and Gel electrophoresis the result was observed under UV illuminator. In the study period from December 2022 to March 2023 among 154 suspected isolates in total, 82 confirmed ***Klebsiella pneumoniae*** isolates were identified after the completion of gel electrophoresis of the PCR products. Among those confirmed isolates 52 isolates were marked from hospital wastewater samples and remaining 29 were marked from the hospital adjacent community tap water. Visualization of the agarose gel electrophoresis under UV illuminator was pasted below.

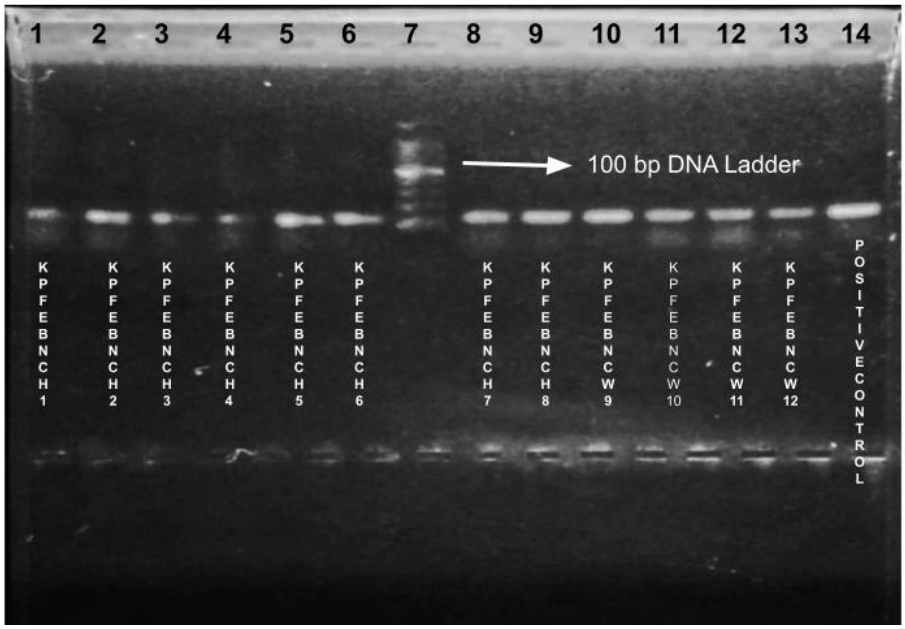
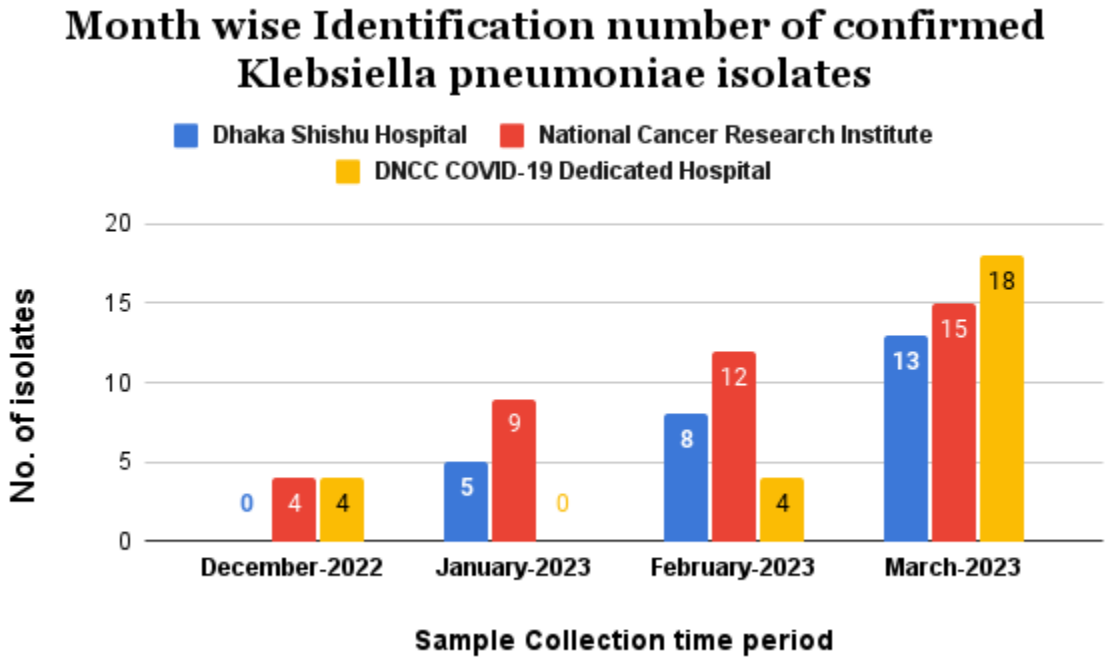


Figure-4: Visualization of the ***Klebsiella pneumoniae*** identification specific PCR

4.3 Month wise Identification of confirmed *Klebsiella pneumoniae* isolates:

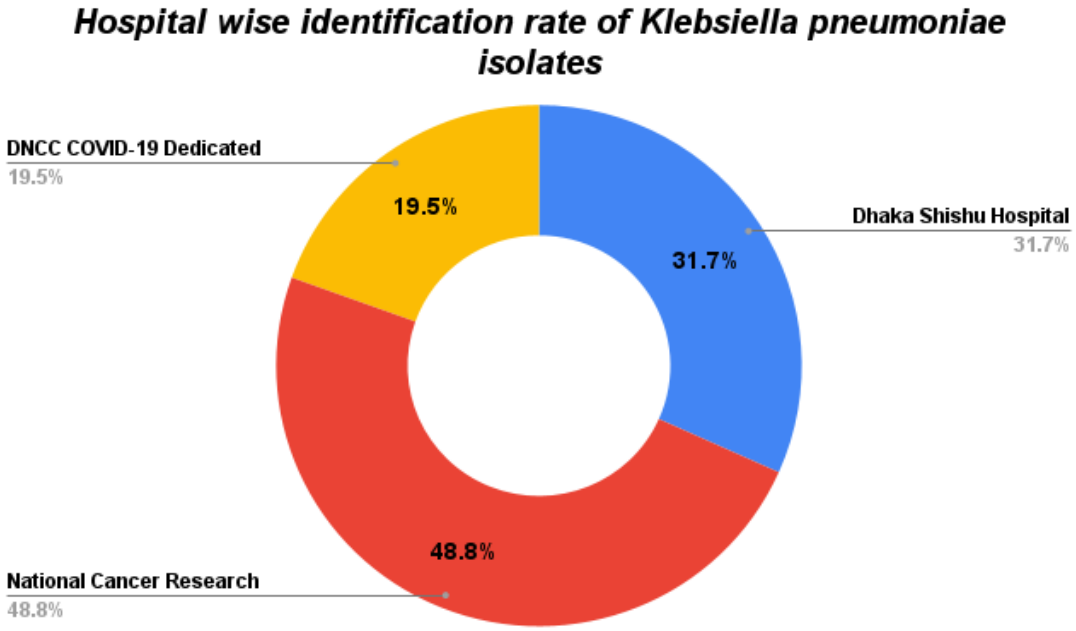
The study period to identify ***Klebsiella pneumoniae*** isolates from hospitals and their adjacent community tap water of Dhaka city was from December 2022 to March 2023 and within this study period from both the hospital and community samples intotal of 82 confirmed ***Klebsiella pneumoniae*** isolates were found. After analyzing the confirmed isolates according to month , it can be seen that on December 2022 the number of the confirmed isolates were 8, on January 2023 the number of the confirmed isolates were 14, on February 2023 the number of the confirmed isolates were 24 and on March 2023 the number of confirmed isolates were 46. So the clear interpretation is that in March 20203 the number of confirmed isolates were the highest and in December 2022 the number of the confirmed isolates were the lowest.



Graph-2: Month wise identification ratio of confirmed *K. pneumoniae* isolates

4.4 Hospital wise identification rate of *Klebsiella pneumoniae* isolates:

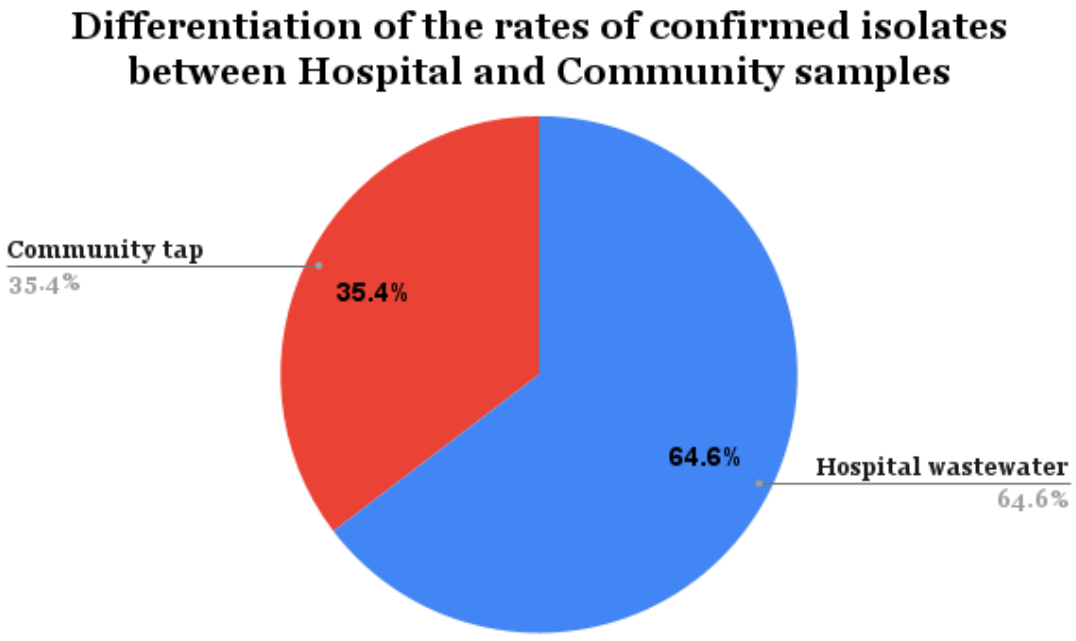
In the study the differentiation of confirmed ***Klebsiella pneumoniae*** isolates was taken into consideration as well. Because the chosen three hospitals were in the most crowded places. Over crowd of people were seen in both hospitals and their adjacent community areas as well. After analyzing the data of identification rate according to hospitals, it can be seen that 48.8% (40 out of 82) from the confirmed isolates were from the National Cancer Research Centre which was the highest in number. On the other hand, rates of confirmed isolates were lowest in DNCC COVID-19 Dedicated hospital which was 19.5% (16 out of 82). Rates of confirmed isolates of Dhaka Shishu Hospital was 31.7% (26 out of 82) which was the second highest rate in number. The visual representation of the rates was given below:



Graph-3: Hospital wise identification rate of ***Klebsiella pneumoniae*** isolates

4.5 Differentiation on the rates of confirmed isolates between Hospital and Community samples:

In the study, a total of 82 isolates of ***Klebsiella pneumoniae*** were identified from 52 samples collected between December-2022 to March-2023. Among those confirmed isolates some were from Hospital wastewater and some were from Community tap water. The ratios among them was also a part of the study to analyze how the rates of the organism variest from the hospital area to community spots. After analyzing the result it was seen that the rate of confirmed isolates were mostly from hospital wastewater samples and the rate was 64.6% (53 out of 82). And from the community tap water samples the identification rate was 35.4% (29 out of 82). The graphical illustration was given below for better understanding.



Graph-4: Differentiation of the rates of confirmed isolates between Hospital and Community samples

4.6 Interpretation of the Antibiotic Susceptibility Testing results:

After completion of the species specific identification, the confirmed ***Klebsiella pneumoniae*** isolates were gone through Antibiotic Susceptibility Testing(AST). In the AST Disk Diffusion method has been followed and 11 antibiotics shown in Table-X were used for identification of ESBL and Carbapenamase resistance ***K. pneumoniae*** isolates. After the incubation period of 18-24 hours, the MHA plates were observed to measure the Zone of Inhibition to identify whether the antibiotics were resistant, sensitive or intermediate for the isolates. The visual representation of the observed plates were shown through the figures.

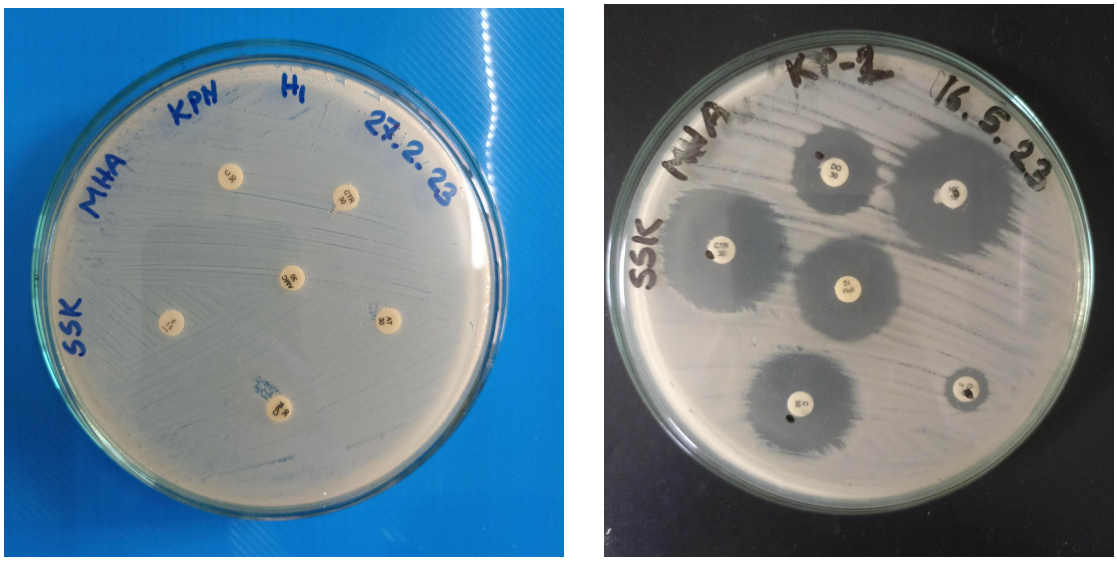
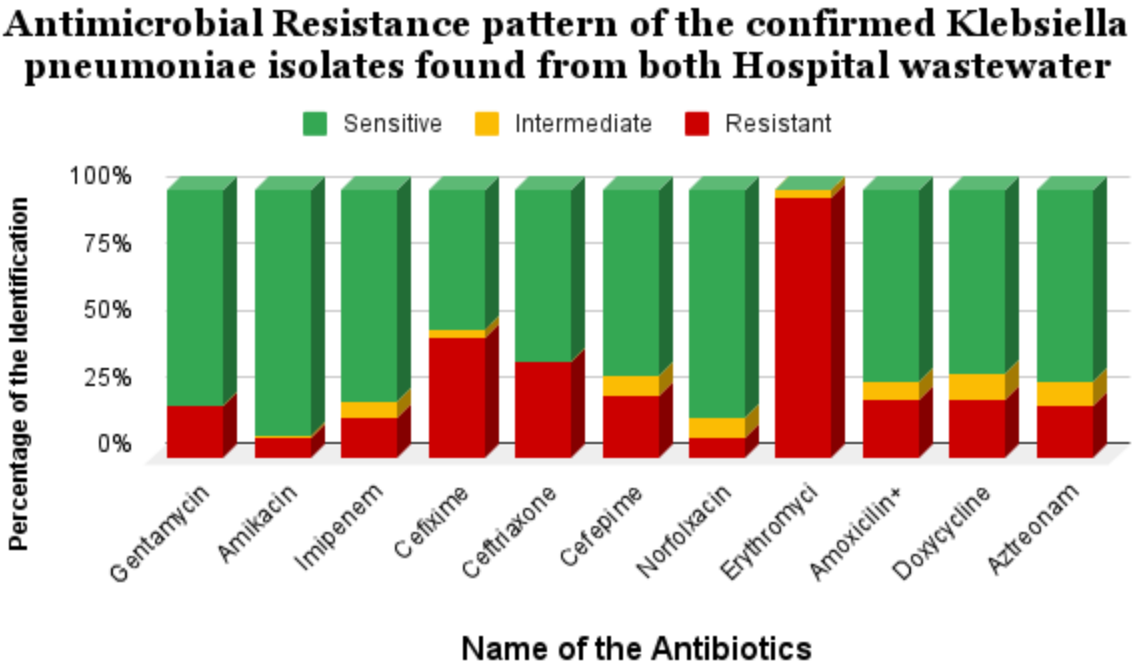


Figure-5: Incubated MHA plates for Antibiotic Susceptibility Testing

4.7 Antimicrobial Resistance pattern of the confirmed *Klebsiella pneumoniae* isolates found from both Hospital wastewater and Community tap water

To identify the ESBL and Carbapenam producers ***Klebsiella pneumoniae*** isolates identified from both hospital wastewater and community tap water AST was done for 11 antibiotics. After the analysis of the AST results it was observed that the Resistance level was quite high for some antibiotics and in all the antibiotics there were resistant zones of inhibition. Among them Erythromycin which was from Marcolids group and the percentage was 97.5% (80 resistant isolates among 82). The second highest was Cefixime which was from 3rd generation Cephalosporin group and the rate was 45.1% (37 resistant isolates from 82).Also, there was resistant isolates for these antibiotics as well and the rates were: 19.5% for Gentamycin, 7.3% for Amikacin, 14.6% for Imipenem, 36.1% for Ceftriaxone, 23.2% for Cefepime, 7.3% for Norfolaxacin, 22.5% for both Amoxicillin+Clavulanic Acid and Doxycycline, 19.5% for Aztreonam. There were resistant isolates in almost all the antibiotics as well but the ratio differs. There were intermediate zones of inhibition for all of 11 antibiotics except for Gentamycin and Ceftriaxone. The highest Intermediate zone of inhibition was seen for Doxycycline and the rate was 9.8% and the lowest one was Amikacin and the rate was 1.2%. And in every isolate the highest rate was for sensitive zones of inhibition except for the Erythromycin. The following graph gives visual representation of the resistant, Intermediate and Sensitivity level for the antibiotics on confirmed isolates.

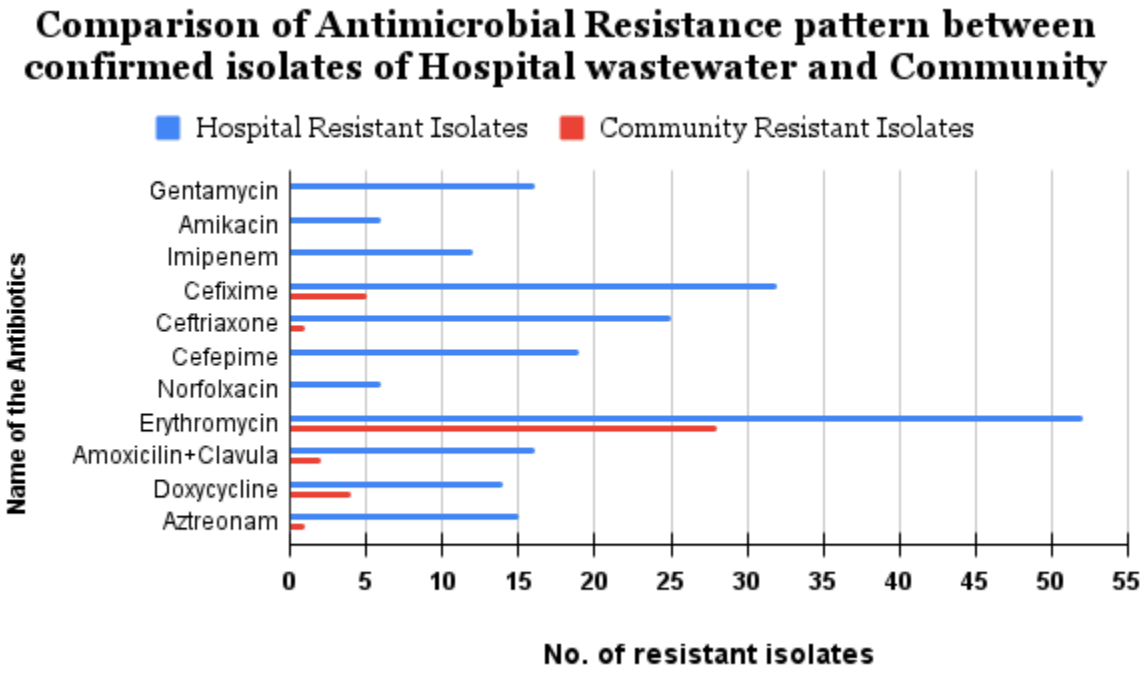


Graph-5: Antimicrobial Resistance pattern of the confirmed ***Klebsiella pneumoniae*** isolates found from both Hospital wastewater and Community tap water

4.8 Comparison of Antimicrobial Resistance pattern between confirmed isolates of Hospital wastewater and Community tap water

After analyzing the data of both hospital confirmed isolates and community confirmed isolates of ***Klebsiella pneumoniae*** it can be seen that the resistant pattern against all the 12 antibiotics was high for Hospital wastewater confirmed isolates and low for the hospital adjacent community tap water. For Gentamycin, Amikacin, Imipenem, Cefepime and Norfloxacin there were no resistant isolates from community tap water samples. The highest rate of resistant isolates from

community tap water was seen for Erythromycin (28 out of 82). For hospital wastewater samples the confirmed isolates were resistant for all the antibiotics and the resistant ratio was high for hospital waste water isolates.



Graph-6: Comparison of Antimicrobial Resistance pattern between confirmed isolates of Hospital wastewater and Community tap water

4.9 Result Interpretation of the MultiDrug Resistance gene identification PCR assay for the selective confirmed isolates:

After analyzing the phenotypic result for the antibiotics, genotypic analysis was carried away. For the genotypic analysis of the 24 selected isolates who were resistant to most of the antibiotics were selected and identification was done for the presence of 8 different MultiDrug Resistance

specific genes which were basically ESBL and Carbapenemase encoded. After analyzing the result it was found that from the selected 24 isolates, 20 isolates give positive results for the selected MDR Genes. After analyzing the specific data it was observed that for SHV the identification rate was high and the rate was 56.7% which was 17 isolates out of 24. Among those 17 isolates 4 were community tap water isolates and remaining 13 isolates were hospital wastewater isolates. For $bla_{\text{CTX-M}}$ the identification rate was 23.3% which was 7 out of 24 isolates and all of them were from hospital wastewater isolates. For $bla_{\text{NDM-1}}$, bla_{TEM} and bla_{KPC} the identification rates were similar and it was 6.7% which was 2 isolates out of 24 isolates and all of them were from hospital wastewater isolates.

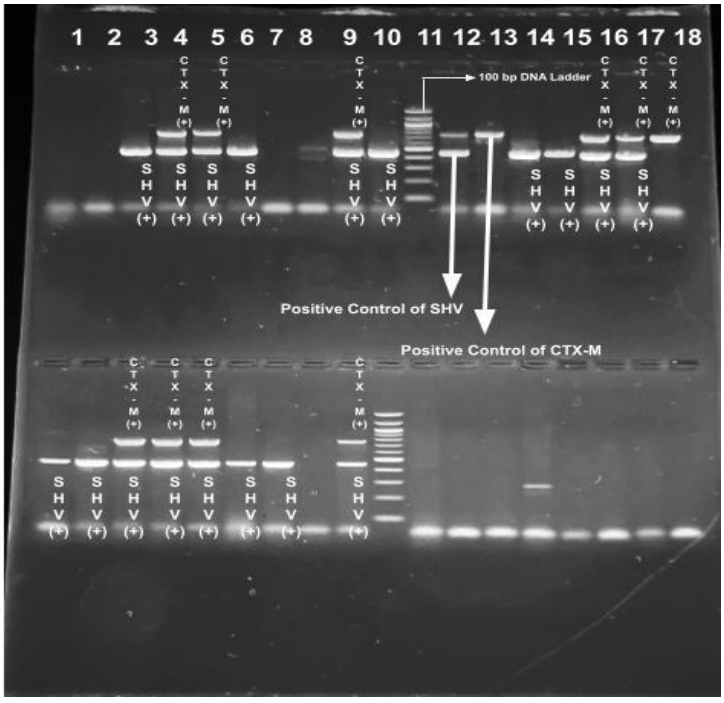
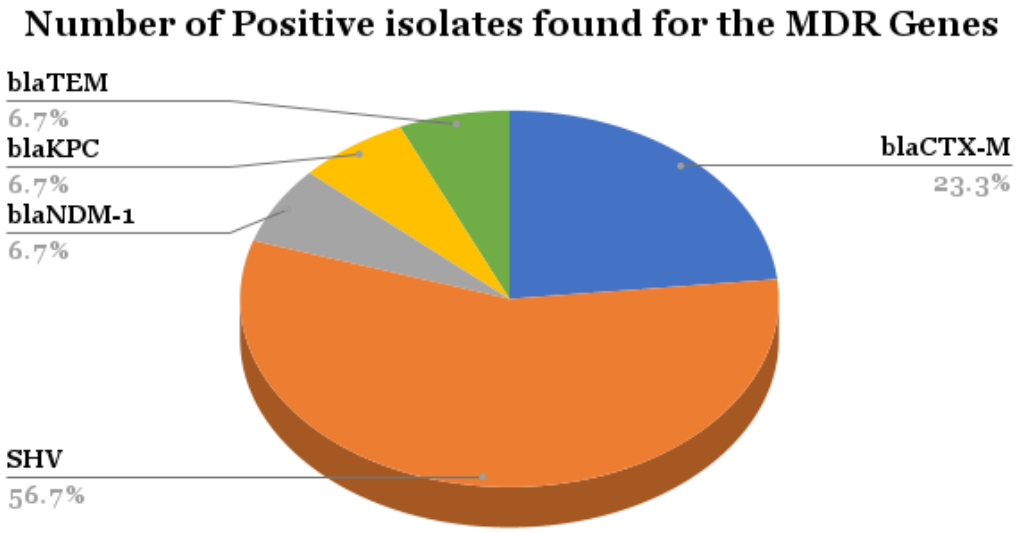


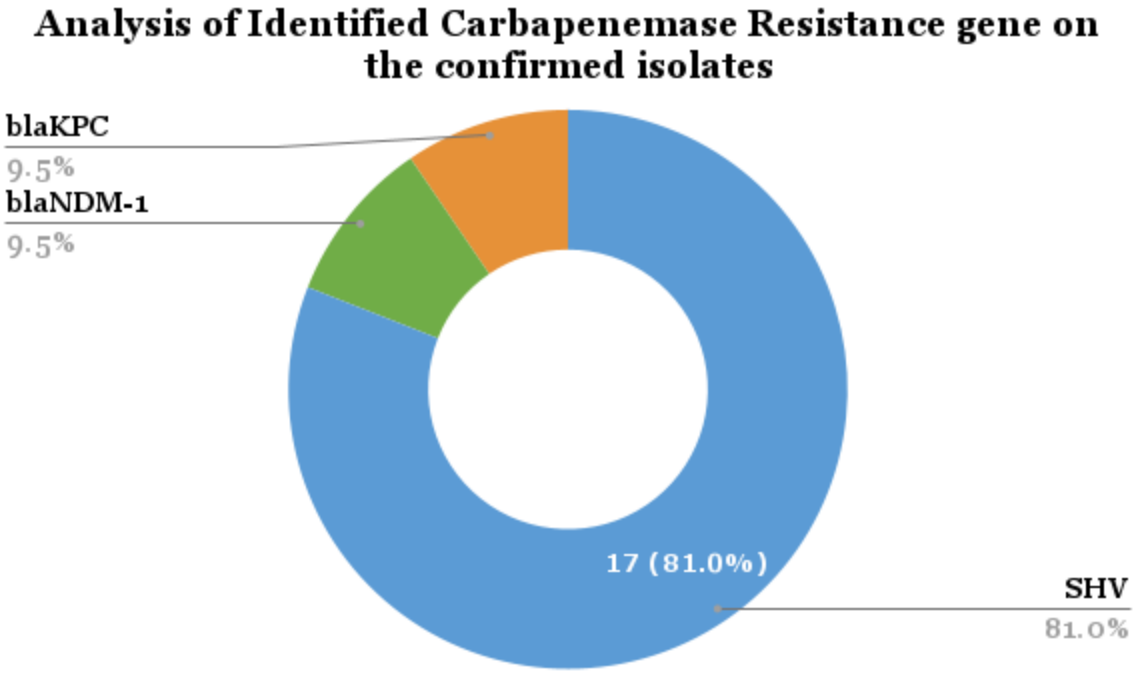
Figure-6: Visualization of MDR gene specific PCR assay



Graph-7: Number of Positive isolates found for the MDR Genes

4.10 Analysis of Identified Carbapenemase Resistance gene on the confirmed isolates:

After the analysis it was observed that there were isolates who had Carbapenamse resistance genes on them. In the study, SHV, $bla_{\text{NDM-1}}$, bla_{KPC}, *these* three gene specific primers were used to identify the carbapenemase resistance gene. If we look into their ratio of having these genes we found out that on 81% of the isolates SHV gives positive results. For both $bla_{\text{NDM-1}}$ and bla_{KPC} 9.5% of the isolates gives positive results. It was also observed that there were 2 isolates from the hospital wastewater which give positive results for both SHV and $bla_{\text{NDM-1}}$.

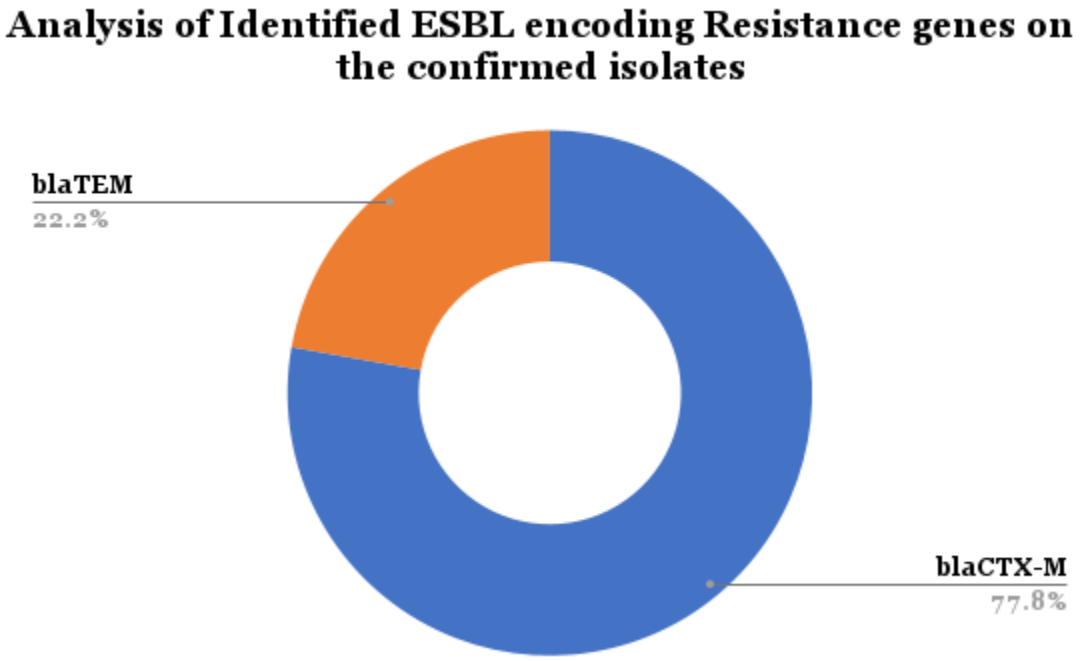


Graph-8: Analysis of Identified Carbapenemase Resistance gene on the confirmed isolates

4.11 Analysis of Identified Extended Spectrum β-Lactamases (ESBL) encoding Resistance genes on the confirmed isolates:

After the analysis it was observed that there were isolates who had ESBL encoding resistance genes on them. In the study, $bla_{\text{CTX-M}}$, bla_{TEM} these two gene specific primers were used to identify the ESBL encoding resistance gene. If we look into their rates of having these genes we found out that on 77.8% (7 out of 24) of the isolates, $bla_{\text{CTX-M}}$ gives positive results. For bla_{TEM} 22.2% (2 out of 24) of the isolates gives positive results. It was also observed that there were no

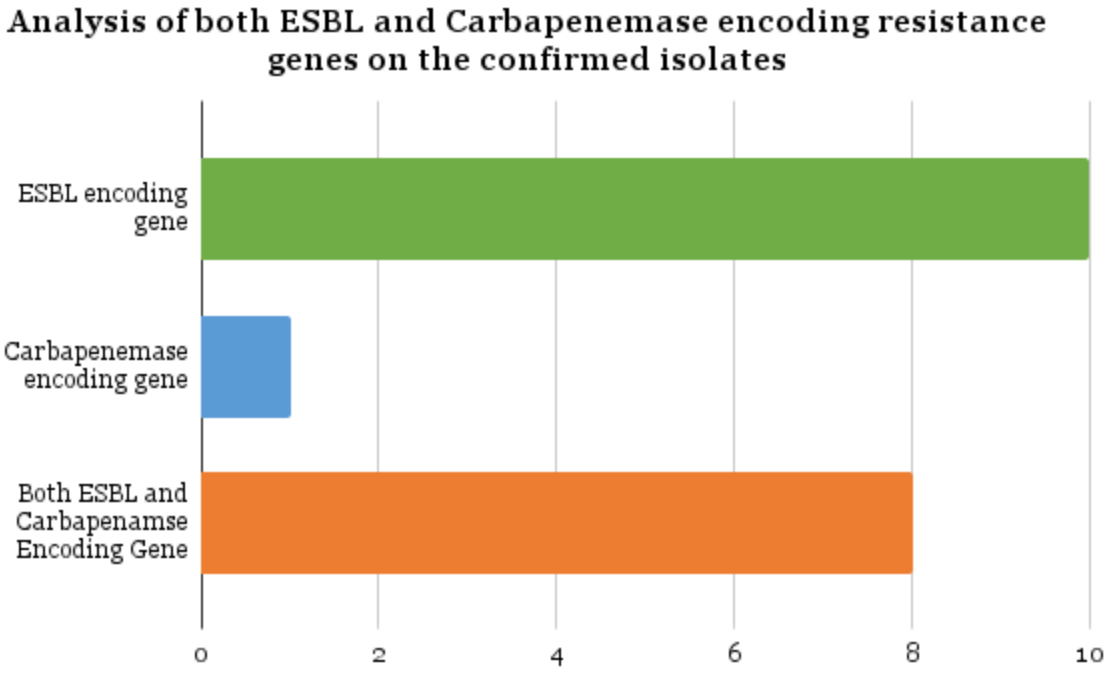
isolates from the hospital wastewater and community tap water which give positive results for both *bla*$_{CTX-M}$ and *bla*$_{TEM}$.



Graph-9: Analysis of Identified ESBL encoding Resistance genes on the confirmed isolates

4.12 Analysis of both ESBL and Carbapenemase encoding resistance genes on the confirmed isolates

After analyzing the whole result of the MultiDrug Resistance Gene it was seen that there were variations in isolates having ESBL, Carbapenemse and both the genes present in them. If the percentage was shown in graph it was observed that among 19 isolates having MDR genes 10 isolates have only ESBL encoding genes and only one isolate have Carbapenemase encoding gene. Remaining 8 isolates contain both ESBL and Crabapenemase encoding genes in them.



Graph-10: Analysis of both ESBL and Carbapenemase encoding resistance genes on the confirmed isolates

CHAPTER-5

DISCUSSION ON THE RESULTS

5.1 Discussion

Globally, ***Klebsiella pneumoniae***, a gram-negative bacterium is a major contributor to Hospital-acquired infections and neonatal sepsis. Widely it is known as an opportunistic pathogen and it can cause a variety of infections in hospitalized patients, most frequently pneumonia, wound, or urinary tract infections. ***K. pneumoniae*** can also be carried asymptomatically by healthy people through intestinal tract, skin, nose and throat (Holt et al., 2015). In the current years, Multidrug Resistant bacterial infections became one of the biggest problems for the doctors and clinicians to treat the infections (Indrajith et al., 2021). Currently, ***K. pneumoniae*** starts to show resistance for the broad spectrum antibiotics like fluoroquinolones and aminoglycosides (Indrajith et al., 2021). Despite being an opportunistic pathogen and a common cause of hospital-acquired infections, ***K. pneumoniae*** can also be found in the non clinical places like the mucosal surfaces of humans and animals as well as in environmental sources like water, soil, sewage and vegetation (Ferreira et al., 2019). In the present study, the target was to find out the ESBL and Carbapenems encoding ***K. pneumoniae*** from the hospital effluent waste waters and their adjacent community tap waters.

From the primary detection, in the study we isolated 82 confirmed ***K. pneumoniae*** isolates from both hospital wastewater and community tap water. But among those 82 isolates 52 were isolated from hospital wastewater and remaining 29 isolates were isolated from hospital adjacent community tap water. The ratios vary from hospital to community because hospital samples were the wastewater of the hospital and community samples were community tap water which was used by local people in their day to day life, the microbial count varies between them because one is wastewater and another is partially treated water. In one study of the Algerian region,

Algeria which was conducted between February 2010 - March 2010, researchers found 52 confirmed ESBL producing ***K. pneumoniae*** isolates from the hospital effluent wastewater (Atmani et al., 2015). After the distribution of confirmed ***K. pneumoniae*** isolates according to the hospital it was seen that from National Cancer Research Institute and Hospital which was situated in Mohakhali, Dhaka maximum number of the confirmed isolates were identified and number was 40 out 82. From Dhaka Shishu Hospital which was situated in Shyamoli, Dhaka number of confirmed isolates were 26 out of 82 which was quite lower than National Cancer Research Institute Hospital. From the DNCC COVID-19 Dedicated Hospital, Mohakhali, Dhaka least number of confirmed isolates were identified and the number was 16 out of 82. Despite being in the same area, the number of isolates varies among National Cancer Research Institute and DNCC COVID-19 Dedicated Hospital can be the varying number of patients admitted in the hospitals. Also, the waste release treatment may vary among both hospitals. In the found data was studied on the basis of Hospital and Community samples it was seen that from Hospital wastewater samples the number of confirmed isolates were higher in number than the community tap water. The number of isolates found from hospital samples were 53 out of 82 which was more than half of the total confirmed isolates and the number of isolates found from the hospital adjacents community tap water were 29 out of 82 which was quite lower in number. Hospital wastewaters were the untreated ones where all the sewage of the hospital from the patients and their attendants. On the other side, community tap water is treated from the wastewater treatment plant so there should be less number of microorganisms present. In our study we observed that the number of microorganisms, especially our target organism ***Klebsiella pneumoniae***, was present in quite high numbers. If we conduct the study for a whole year we

may also observe the differentiation of the number of isolates between hospital wastewater and community tap water samples.

Antibiotic Resistance became a threat for the whole world as it became one of the causes of major health issues. To treat diseases caused by ***Enterobacteriaceae*** which includes ***Klebsiella pneumoniae*** broad spectrum Beta-lactam antibiotics have been used since the 1950s (Lepuschitz et al., 2019). Due to overuse of these antibiotics it became a threat in present days. In the recent years, spreading of MultiDrug Resistant ***Klebsiella pneumoniae*** has been raised due to poor infrastructure of the wastewater purifying plants and overuse of antibiotics by the clinicians. This negligence is putting the public health of a country at a high risk. Also, increased use of antibiotics to cure diseases raised the chance of resistant gene acquisition in ***K. pneumoniae*** by horizontal gene transfer method. This serious issue arises just because of improper treatment of the wastewaters released by the hospitals into the environment.

In our study our target was to find out ESBL encoding and Carbapenem resistant ***Klebsiella pneumoniae*** from both the hospital wastewater and their adjacent community tap waters. During our study period from December 2022 to March 2023 we confirmed 82 isolates and all of them were either ESBL encoding or Carbapenem resistant or both ***K. pneumoniae***. To identify the antibiotic resistance pattern phenotypically Disk Diffusion Antibiotic Susceptibility testing was done. In the AST, we used 12 antibiotics and among them the most resistant were: Erythromycin(97.5%), Cefixime(45.1%), Ceftriaxone(36.1%), Cefepime(23.2%), Amoxicillin & Clavulanic acid(22.5%), Doxycycline(22.5%) and Aztreonam(19.5%). For Amikacin(7.3%), Gentamicin(19.5%), Imipenem(14.6%) and Norfloxacin(7.3%) antibiotics, the number of

resistant isolates was low in number. Among the 12 antibiotics Erythromycin was the only isolate which gave a resistant zone of inhibition in 81 isolates out of 82 isolates.

K. pneumoniae isolates isolated from hospital wastewater samples gave resistance patterns for all of the antibiotics and the highest rate was for Erythromycin and lowest rate were for Norfloxacin and Amikacin. Isolates of ***K. pneumoniae*** isolated from hospital adjacent community water samples gave a resistant pattern for Erythromycin, Cefexime, Doxycycline, Ceftriaxone, Amoxicillin and Clavuclanic acid and Aztreonam. Among them the highest rate was for Erythromycin. For the remaining antibiotics community isolates gave a sensitive zone of inhibition pattern.

After completion of the phenotypical analysis among those 82 confirmed ***K. pneumoniae*** isolates by seeing their AST pattern 24 isolates were selected from both hospital wastewater and community tap water samples for the identification of gene which encodes for ESBL and Carabapenem resistance. To identify the ESBL encoding, ***K. pneumoniae*** isolates $bla_{\text{CTX-M}}$ and bla_{TEM} genes were screened through the $bla_{\text{CTX-M}}$ and bla_{TEM} specific primers. For $bla_{\text{CTX-M}}$ only 7 isolates give positive results and for bla_{TEM} only 2 isolates out of 24 give positive results for this gene. Among these isolates positive for both genes, all of them were from hospital wastewater samples and none of them were from community tap water. To identify the Carbapenem resistant isolates SHV, $bla_{\text{NDM-1}}$, bla_{KPC} genes were searched through these gene specific primers base PCR. Among these genes for SHV 17 isolates gives positive results among 24 isolates. For $bla_{\text{NDM-1}}$ and bla_{KPC},only 2 isolates give positive results. From the 17 positive isolates for SHV there were 4 isolates which were positive from community tap water samples. There were

multiple isolates which gave positive results for both ESBL and Carbapenem resistant ***K. pneumoniae*** isolates and the number of those isolates were 8. Those 8 isolates were isolated from hospital wastewater samples, none of them were from community tap water.

In our study, we found mostly the Carbapenem resistant ***K. pneumoniae*** isolates and most of them were from hospital wastewater. Our target was to find out the same kind of resistant ***K. pneumoniae*** isolates from both the hospital wastewater and community tap water. But as per our findings we found confirmed isolates from both hospital wastewater and community tap water but presence of the same kind of resistance gene varies between them.

In one study of Bangladesh it was seen that from hospital patients and hospital environments 67 of the *K. pneumoniae* isolates were Resistant to Multiple drugs and 42 were Carbapenem resistant which were confirmed by gene specific PCR assay. These isolates also formed biofilms (Mahmud et al., 2022). This study shows that the isolates identified from hospital patients were Multi Drug Resistant and those MDR isolates were passed to the environment through their caregivers or their used materials. This poses a great threat to the public health of this country if these transmissions go on for a long time. In our study our motive was also to find out the relationship among the isolates of hospital wastewaters and their adjacent community water. Maybe for some reason we could not find out our definitive results as per our motive. But the main motive was to let clinicians be aware of the fact that overuse of antibiotics will harm both the patients and its environment in many ways in the near future.

In one study of USA conducted in 2019, researchers found out that from both the public and private tap water there were positive isolates of *Enterobacteriaceae* including ***K. pneumoniae*** which have ESBL categorized bla_{SHV} or bla_{TEM}, or bla_{OXA-48}-type carbapenemase or bla_{CTX-M} genes (Tanner et al., 2019). In a developed country like the USA researchers find ESBL producing and Carbapenem resistant strains of ***K. pneumoniae*** from the public and private drinking water which signifies a biggest concern on Antibiotic resistance. But in our study conducted in Dhaka, Bangladesh which is an overpopulated and Developing country's city and also despite sampling from hospital adjacent community tap water the rates of positive ESBL encoding and Carbapenam resistant ***K. pneumoniae*** was almost zero. It may be possible if the study conducted for over a year than the suspected isolates may be identified higher in number. Still, the number of isolates found from hospital wastewater samples which were ESBL encoded and Carbapenem resistant are quite remarkable. Further studies can be done on them.

5.2 Limitations of the Study

This whole study was conducted on a particular area which was three hospitals from Dhaka North City Corporation for a study on the particular location. If both south and north city corporations of Dhaka city were taken into consideration then a more specific and visible scenario can be seen. In this study only microbiological analysis was seen but if the study focused on both microbiological and chemical analysis then a whole scenario can be analysed. In that scenario chemical elements, toxic materials and microorganisms all can be seen from the study spots. Also, unavailability of different Antibiotics antibiotics from different classes cannot be analyzed. There must be other classes of antibiotics for which the isolates may give a resistant pattern as well. Moreover, due to the unavailability of the materials to identify whether the

isolates were pathogenic or not we could not interpret our results on the pathogenicity basis as well. If the MDR gene associated isolates were found pathogenic from the environment this will be a great threat for the public health of Dhaka city's people.

CHAPTER-6

CONCLUSION

The successful transmission of microbes from hospital effluents to adjacent community tap water indicates the mismanagement of hospital waste. Moreover, ***Klebsiella pneumoniae*** has been notorious for spreading serious infections like nosocomial infections, pneumonia, and UTIs. In the recent decade, patients with pneumonia are increasing where antibiotic resistance in the bacteria is observed. Over time usage of various classes of antibiotics has made the strains drug tolerant. It has been observed that even the last line of antibiotics such as Carbapenems are resisted by ***K. pneumoniae***. This creates serious difficulty in treatment plans especially for patients who are immunosuppressed and prone to illness.

From our study of 82 confirmed ***K. pneumoniae*** isolates, 24 isolates were selected based on the phenotypic characteristics of the Antimicrobial Susceptibility Test (AST). Among these 24 isolates, a total of 20 isolates were positive for various MDR genes. 16 positive isolates were acquired from hospital effluents while the remaining (4) positive isolate was from the community tap water. If a ratio of the 20 positive isolates is shown, 23.3% were positive for $bla_{\text{CTX-M}}$ and 6.7% were positive for bla_{TEM}, bla_{KPC}, and $bla_{\text{NDM-1}}$ genes respectively. Moreover, for the SHV gene, 56.7% of isolates were positive. This ratio indicates that the bacteria ***K. pneumoniae*** acquired from both the hospital effluent and community tap water retains the ability to resist multiple classes of antibiotics.

From the findings, it is clear that most hospital-acquired isolates are resistant to multiple antibiotics which creates difficulty in treatment for serious diseases. Also, resistance in community tap water isolates show the possibility of antibiotic-resistant gene transfer via horizontal gene transfer. In this study, an association between multi-drug resistance, hospital and

community isolates along with poor management of wastewater treatment is present. Due to the widespread antibiotic resistance to environmental bacteria, strict surveillance and management of waste are crucial. Our study was conducted in a limited area of three hospital settings and in a limited time. It is important to conduct further studies and research on how to combine different spectrums of antibiotics to lessen the possible consequences of getting infected by multidrug resistant ***Klebsiella pneumoniae.***

CHAPTER-7

APPENDIX

7.1 Appendix-1

Media Composition

MacConkey Agar

Commercial name: HIMEDIA® MacConkey Agar w/0.15% Bile salts, CV & NaCl

Component	Amount (Gms/liter)
Peptone	1.50
Tryptone	1.50
Gelatine Peptone	17.00
Lactose	10.00
Bile salts	1.50
Sodium Chloride	5.00
Crystal Violet	0.001
Neutral Red	0.03
Agar	15.00

*Final pH (at 25℃) 7.1±0.2

Nutrient Agar

Commercial name: TM-MEDIA Nutrient Agar

Components	Amount (gms/liter)
Agar	15.0
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5

*Final pH (at 25℃) 7.4±0.2

Mueller Hinton Agar

Commercial Name: HIMEDIA® Mueller Hinton Agar

Components	Amount (gms/liter)
HM infusion B form*	300.00
Acicase™	17.50
Starch	1.50
Agar	17.00

*Final pH (at 25℃) 7.3±0.1

Buffered Peptone Water

Components	Amount (gms/liter)
Peptone / Tryptone	10.0
Sodium Chloride	5.0
Disodium phosphate	3.5
Mono-potassium phosphate	1.5

*Final pH (at 25℃) 7.2 ± 0.2.

Tris-EDTA Buffer(TBE) 10X (Stock)

Components	Amount
Tris Base	108gm
Boric Acid	55gm
Double distilled water	900mL
0.5M EDTA (pH=8.0)	40mL

*Adjust volume to 1 liter

1X Tris-EDTA Buffer (TBE)

Components	**Amount**
Tris Base (pH= 7.6)	0.13 M
Boric Acid	4.5mM
EDTA	2.5mM

CHAPTER-8

REFERENCES

1. Agarwal, A., Srivastava, J., Maheshwari, U., & Iftikhar, M. (2018). Molecular characterization and antimicrobial susceptibility profile of New Delhi metallo-beta-lactamase-1-producing escherichia coli among hospitalized patients. Journal of Laboratory Physicians, 10(02), 149–154. https://doi.org/10.4103/jlp.jlp_76_17
2. Ashrust, J. V., & Dawson, A. (2023). Klebsiella Pneumonia. Treasure Island (FL): StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK519004/#_article-23903_s2_
3. Atmani, S. M., Messai, Y., Alouache, S., Fernández, R., Estepa, V., Torres, C., & Bakour, R. (2015). Virulence characteristics and genetic background of ESBL-producing Klebsiella pneumoniae isolates from wastewater. Fresenius Environmental Bulletin, 24(1), 103-112.
4. Cruz-Córdova, A., Esteban-Kenel, V., Espinosa-Mazariego, K., Ochoa, S. A., Moreno Espinosa, S., de la Garza Elhain, A., Fernández Rendón, E., Oliver López Villegas, E., & Xicohtencatl-Cortes, J. (2014, January 1). Pathogenic determinants of clinical klebsiella pneumoniae strains associated with their persistence in the hospital environment. Boletín Médico del Hospital Infantil de México. https://www.elsevier.es/es-revista-boletin-medico-del-hospital-infantil-401-articulo-pathogenic-determinants-clinical-klebsiella-X1665114614779244
5. Coorevits, L., Boelens, J., & Claeys, G. (2015). Direct susceptibility testing by disk diffusion on clinical samples: A rapid and accurate tool for antibiotic stewardship.

European Journal of Clinical Microbiology & Infectious Diseases, 34(6), 1207–1212. https://doi.org/10.1007/s10096-015-2349-2

6. Cordova-Espinoza, M. G., Giono-Cerezo, S., Sierra-Atanacio, E. G., Escamilla-Gutierrez, A., Carrillo-Tapia, E., Carrillo-Vazquez, L. I., Mendoza-Perez, F., Leyte-Lugo, M., Gonzalez-Vazquez, R., Mayorga-Reyes, L., & Gonzalez-Vazquz, R. (2023, April 23). Isolation and Identification of Multidrug-Resistant Klebsiella pneumoniae Clones from the Hospital Environment. Pathogens, 12(5), 634. MDPI. https://doi.org/10.3390/pathogens12050634
7. Diaz, M. H., Waller, J. L., Napoliello, R. A., Islam, Md. S., Wolff, B. J., Burken, D. J., Holden, R. L., Srinivasan, V., Arvay, M., McGee, L., Oberste, M. S., Whitney, C. G., Schrag, S. J., Winchell, J. M., & Saha, S. K. (2013). Optimization of multiple pathogen detection using the Taqman Array Card: Application for a population-based study of neonatal infection. PLoS ONE, 8(6). https://doi.org/10.1371/journal.pone.0066183
8. Denissen, J., Reyneke, B., Waso-Reyneke, M., Havenga, B., Barnard, T., Khan, S., & Khan, W. (2022, July 13). Prevalence of ESKAPE pathogens in the environment: Antibiotic resistance status, community-acquired infection and risk to human health. *International Journal of Hygiene and Environmental Health*, *244*. ScienceDirect. https://doi.org/10.1016/j.ijheh.2022.114006
9. Doosti, A., Pourabbas, M., Arshi, A., Chehelgerdi, M., & Kabiri, H. (2015). TEM and SHV genes in Klebsiella pneumoniae isolated from cockroaches and their antimicrobial resistance pattern. Osong Public Health and Research Perspectives, 6(1), 3–8. https://doi.org/10.1016/j.phrp.2014.10.011

10. Elazhary, M. A., Saheb, S. A., Roy, R. S., & Lagacé, A. (1973). A simple procedure for the preliminary identification of aerobic gram negative intestinal bacteria with special reference to the Enterobacteriaceae. Canadian journal of comparative medicine : Revue canadienne de medecine comparee, 37(1), 43–46.
11. Ferreira, R. L., Da Silva, B. C. M., Rezende, G. S., Nakamura-Silva, R., Pitondo-Silva, A., Campanini, E. B., Brito, M. C. A., Da Silva, E. M. L., De Melo Freire, C. C., Da Cunha, A. F., & Da Silva Pranchevicius, M. C. (2019). High prevalence of multidrug-resistant klebsiella pneumoniae harboring several virulence and β-lactamase encoding genes in a brazilian intensive care unit. Frontiers in Microbiology, 10(JAN). https://doi.org/10.3389/fmicb.2018.03198
12. Filis, P. E. L., Cholley, P., Gbaguidi-Haore, H., Hocquet, D., Sauget, M., & Bertrand, X. (2021, March 24). ESBL-producing Klebsiella pneumoniae in a University hospital: Molecular features, diffusion of epidemic clones and evaluation of cross-transmission. PLOS ONE, 16(3). https://doi.org/10.1371/journal.pone.0247875
13. Galarde-López, M., Velazquez-Meza, M. E., Bobadilla-del-Valle, M., Carrillo-Quiroz, B. A., Cornejo-Juárez, P., Ponce-de-León, A., Sassoé-González, A., & Alpuche-Aranda, C. M. (2022). Surveillance of antimicrobial resistance in hospital wastewater: Identification of carbapenemase-producing Klebsiella spp.. Antibiotics, 11(3), 288. https://doi.org/10.3390/antibiotics11030288
14. Gupta, N. (2019). DNA extraction and polymerase chain reaction. Journal of Cytology, 36(2), 116. https://doi.org/10.4103/joc.joc_110_18
15. Gurung, S., Kafle, S., Dhungel, B., Adhikari, N., Thapa Shrestha, U., Adhikari, B., Banjara, M. R., Rijal, K. R., & Ghimire, P. (2020). <P>detection of oxa-48

gene in carbapenem-resistant <EM>escherichia coli and klebsiella pneumoniae from urine samples</p>. Infection and Drug Resistance, Volume 13, 2311–2321. https://doi.org/10.2147/idr.s259967

16. Holt, K. E., Wertheim, H., Zadoks, R. N., Baker, S., Whitehouse, C. A., Dance, D., Jenney, A., Connor, T. R., Hsu, L. Y., Severin, J., Brisse, S., Cao, H., Wilksch, J., Gorrie, C., Schultz, M. B., Edwards, D. J., Nguyen, K. V., Nguyen, T. V., Dao, T. T., … Thomson, N. R. (2015). Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in   klebsiella pneumoniae   , an urgent threat to public health. Proceedings of the National Academy of Sciences, 112(27). https://doi.org/10.1073/pnas.1501049112
17. Indrajith, S., Mukhopadhyay, A. K., Chowdhury, G., Farraj, D. A., Alkufeidy, R. M., Natesan, S., Meghanathan, V., Gopal, S., & Muthupandian, S. (2021). Molecular insights of carbapenem resistance Klebsiella pneumoniae isolates with focus on multidrug resistance from clinical samples. Journal of Infection and Public Health, 14(1), 131–138. https://doi.org/10.1016/j.jiph.2020.09.018
18. Khosravi, A. D., & Mihani, F. (2008). Detection of metallo-β-lactamase–producing pseudomonas aeruginosa strains isolated from burn patients in Ahwaz, Iran. Diagnostic Microbiology and Infectious Disease, 60(1), 125–128. https://doi.org/10.1016/j.diagmicrobio.2007.08.003
19. Lepuschitz, S., Schill, S., Stoeger, A., Pekard-Amenitsch, S., Huhulescu, S., Inreiter, N., Hartl, R., Kerschner, H., Sorschag, S., Springer, B., Brisse, S., Allerberger, F., Mach, R. L., & Ruppitsch, W. (2019). Whole genome sequencing reveals resemblance

between Esbl-producing and carbapenem resistant Klebsiella pneumoniae isolates from Austrian rivers and clinical isolates from hospitals. Science of The Total Environment, 662, 227–235. https://doi.org/10.1016/j.scitotenv.2019.01.179

20. Li, D., Huang, X., Rao, H., Yu, H., Long, S., Li, Y., & Zhang, J. (2023, April 20). Klebsiella pneumoniae bacteremia mortality: a systematic review and meta-analysis. *Cellular and Infection Microbiology*, *13*. Frontiers. https://doi.org/10.3389/fcimb.2023.1157010
21. Mahmoud, N. E., Altayb, H. N., & Gurashi, R. M. (2020). Detection of carbapenem-resistant genes in escherichia coli isolated from drinking water in Khartoum, Sudan. Journal of Environmental and Public Health, 2020, 1–6. https://doi.org/10.1155/2020/2571293
22. Mahmud, Z. H., Uddin, S. Z., Moniruzzaman, M., Ali, S., Hossain, M., Islam, Md. T., Costa, D. T., Islam, M. R., Islam, Md. S., Hassan, Md. Z., Ong, L.-A., Moore, C. E., Charles, K. J., Mondal, D., Lopes, B. S., & Parveen, S. (2022). Healthcare facilities as potential reservoirs of antimicrobial resistant Klebsiella pneumoniae: An emerging concern to public health in Bangladesh. Pharmaceuticals, 15(9), 1116. https://doi.org/10.3390/ph15091116
23. National Institute of Health. (2020). M100[pdf]. Clinical and Laboratory Standards Institute. https://www.nih.org.pk/wp-content/uploads/2021/02/CLSI-2020.pdf
24. Otaigbe, I. I., & Elikwu, C. J. (2023, May 31). Drivers of inappropriate antibiotic use in low- and middle-income countries. *JAC-Antimicrobial Resistance*, *5*(3). Oxford Academic. https://doi.org/10.1093/jacamr/dlad062

25. Paczosa, M. K., & Mecsas, J. (2016, June 15). Klebsiella pneumoniae: Going on the Offense with a Strong Defense. Microbiology and Molecular Biology Reviews, 80(3), 629-661. American Society of Microbiology. https://doi.org/10.1128/mmbr.00078-15
26. Pitout, J. D.D., Nordmann, P., & Poirel, L. (2015, July 13). Carbapenemase-Producing Klebsiella pneumoniae, a Key Pathogen Set for Global Nosocomial Dominance. Antimicrobial Agents and Chemotherapy, 59(10), 5873-5884. American Society of Microbiology. https://doi.org/10.1128/aac.01019-15
27. Rahman, S. u., Ali, T., Ali, I., Khan, N. A., Han, B., & Gao, J. (2018, March 26). The Growing Genetic and Functional Diversity of Extended Spectrum Beta-Lactamases. BioMed Research International, 2018. https://doi.org/10.1155/2018/9519718
28. Ripon, R. K., Motahara, U., Ahmed, A., Devnath, N., Akter Mahua, F., Hashem, R. B., Ishadi, K. S., Alam, A., Sujan, M. S. H., & Sarkar, M. S. (2023, May 31). Exploring the prevalence of antibiotic resistance patterns and drivers of antibiotics resistance of Salmonella in livestock and poultry-derived foods: a systematic review and meta-analysis in Bangladesh from 2000 to 2022. *JAC-Antimicrobial Resistance*, *5*(3). Oxford Academic. https://doi.org/10.1093/jacamr/dlad059
29. Tanner, W. D., VanDerslice, J. A., Goel, R. K., Leecaster, M. K., Fisher, M. A., Olstadt, J., Gurley, C. M., Morris, A. G., Seely, K. A., Chapman, L., Korando, M., Shabazz, K.-A., Stadsholt, A., VanDeVelde, J., Braun-Howland, E., Minihane, C., Higgins, P. J., Deras, M., Jaber, O., … Gundlapalli, A. V. (2019). Multi-state study of Enterobacteriaceae harboring extended-spectrum beta-lactamase and carbapenemase genes in U.S. drinking water. Scientific Reports, 9(1). https://doi.org/10.1038/s41598-019-40420-0

30. Valenzuela-Valderrama, M., González, I. A., & Palavecino, C. E. (2019, September 7). Photodynamic treatment for multidrug-resistant Gram-negative bacteria: Perspectives for the treatment of Klebsiella pneumoniae infections. Photodiagnosis and Photodynamic Therapy, 28, 256-264. ScienceDirect. https://doi.org/10.1016/j.pdpdt.2019.08.012.

31. Wang, X., Bi, C., Xin, X., Zhang, M., Fu, H., Lan, L., Wang, M., & Yan, Z. (2023, May 8). Pyroptosis, apoptosis, and autophagy are involved in infection induced by two clinical Klebsiella pneumoniae isolates with different virulence. Cellular and Infection Microbiology, 13. Frontiers. https://doi.org/10.3389/fcimb.2023.1165609

32. Wyres, K. L., Lam, M. M.C., & Holt, K. E. (2020, February 13). Population genomics of Klebsiella pneumoniae. Nature Reviews Microbiology, 18(6), 344-359. Nature. https://doi.org/10.1038/s41579-019-0315-1

33. Zhang, Y.-L., Huang, F.-Y., Gan, L.-L., Yu, X., Cai, D.-J., Fang, J., Zhong, Z., Guo, H., Xie, Y., Yi, J., Wang, Z., & Zuo, Z.-C. (2021). High prevalence of blactx-M and blaSHV among ESBL producing E. coli isolates from beef cattle in China's sichuan-chongqing circle. Scientific Reports, 11(1). https://doi.org/10.1038/s41598-021-93201-z

34. Zhang, Q., Gao, H.-Y., Li, D., Li, Z., Qi, S.-S., Zheng, S., Bai, C.-S., & Zhang, S.-H. (2019). clinical outcome of escherichia coli bloodstream infection in cancer patients with/without biofilm formation: A single-center retrospective study. Infection and Drug Resistance, Volume 12, 359–371. https://doi.org/10.2147/idr.s192072

THE END