Isolation and Characterization of *Klebsiella pneumoniae* from medical waste and adjoining community water

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This Thesis Dissertation Paper is Submitted in Partial Fulfillment of The Requirement for The Degree Completion of Bachelor of Science in Microbiology to The Department of Mathematics and Natural Sciences, BRAC University.

Microbiology Program

Department of Mathematics and Natural Science

BRAC UNIVERSITY

March, 2023

Declaration

It is hereby declared that-

1. The thesis submitted is our own original work while completing the 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.

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Dedicated To

From the core of our heart, this research work is dedicated to our parents & our family for their continuous and amazing support. And of course, with all gratitude we would like to thank Almighty for providing us with strength and patience to keep our soul consistent with all the hard times.

Ethical Statement

This research is conducted under the appropriate supervision; thus, it is the original work of the author. During these tests, no living being were harmed in any way. The paper is written in such a way that it does not contain any information that has been published or written by a third party in the past, with the exception of instances in which this information is appropriately cited through the use of full and accurate referencing. The experiment was executed in accordance with all of the guidelines and restrictions outlined by the Natural Sciences laboratory inside the Department of Mathematics and Natural Sciences at BRAC University.

Acknowledgment

We acknowledge our gratitude to the honorable **Professor A F M Yusuf Haider**, Chairperson of the MNS Department of BRAC University, for allowing us to complete our undergraduate thesis.

Sincere appreciation goes to our dear Supervisor, senior lecturer **Md. Hasanuzzaman** of BRAC University's MNS Department for accepting us as thesis students and allowing us to complete the graduation. Also, we would like to thank **Akash Ahmed,** senior lecturer, for his co-operation and supportive instructions throughout the whole journey.

We would like to extend our appreciation to the respective **lab officers** and research assistants **Aziz Hossain Shovon**, **Namira Nusrat** and **Nishat Tasnim Ananna** for their suggestions and guidelines during our lab functions and performence.

We would also like to thank **everyone** in our group for everything they have done to help, inspire and keep things in order even though we fell over and over again but we stood up and found our way out to stand on our feet.

Lastly, we want to thank our **family** for their encouragement and their unconditional support throughout our life.

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List of Acronyms

KPC	Klebsiella pneumoniae carbapenemase
ESBL	Extended Spectrum Beta-Lactamase
NA	Nutrient agar
BPW	Buffered Peptone Water
PCR	Polymerase chain reaction
MHA	Mueller-Hinton agar
AST	Antibiotic Sensitivity Test
TE	Tris-EDTA
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
EDTA	Ethylenediamine tetra acetic acid
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
XDR	Extensively drug-resistant
MDR	Multidrug-resistant
PDR	Pan Drug-resistant
NICRH	National Institute of Cancer Research & Hospital
DSH	Dhaka Shishu (Children) Hospital
CLSI	Clinical and Laboratory Standards Institute

EIA	Environmental impact assessment
UTI	Urinary tract infection
AMR	Antimicrobial Resistance
UV	Ultra violet
KPN	*Klebsiella pneumoniae*
SPN	*Streptococcus pneumoniae*
DH	District Hospital
MCWC	Mother and Child Welfare center

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Abstract

This study is designed to conduct an experiment by following a standard protocol established according to the lab facilities and resources which is focused on the highly rising cause of respiratory infection of humans as well as an increasing risk factor at the sector of acquiring multidrug resistance genes and becoming a challenge to treat patients with severe conditions or different types of diseases at the same time. In this study the highly known Carbapenem and beta lactamase producing Klebsiella pneumoniae has been given the priority to experiment the fact that how much resistance and sensitivity is related to this organism in the context of Bangladesh, especially in highly polluted and unhygienic condition of Dhaka city. Therefore, to conduct this experiment three different locations have been selected primarily to observe the pattern of *Klebsiella pneumoniae* in the hospital wastewater and adjacent communities around those hospitals on a yearly basis which is conducted from June to November for our group. The main objective of this study was to observe and monitor if there is any correlation or connection among the strains of *Klebsiella pneumoniae* that can cause a threat of resistance towards multiple antibiotics specially the carbapenems (Meropenem, Imipenem etc.) and other antibiotics of different generation. Also, it was operated to gain the idea about how likely the chances are for this organism to gain resistance genes from different regions of hospitals and their nearby communities. This hypothesis of correlation is regarded as an experiment which does not only focus on the resistance pattern but also the fact that, if there is any chance of any kind of outbreak from this type of correlation or so on. Furthermore, this experiment focused on more specific and confirmatory methods for identification and confirmation of target organism *Klebsiella pneumoniae* which is PCR (Polymerase chain reaction) and ATCC strains are used as positive controls. Since biochemical methods are not that accurate like PCR and also require time and resources, these methods are kept aside to continue the study in a standard procedure. Moreover, antimicrobial susceptibility tests were performed to check the resistance and sensitivity towards different antibiotics which will be described in the methods and result section.

The quality of drinking water and the distribution systems as well as waste management of hospital water has now become one of the major concerning issues for spreading antimicrobial resistance among different organisms specially for ubiquitous organisms like Klebsiella pneumoniae. (Giri et al., 2021) Since this is an emerging problem for public health it has become an unavoidable concern to test the presence of antibiotics and organisms that may develop resistance against those available antibiotics. (Armstrong et al., 1981, Schwartz et al., 2003). Although several researches have been conducted relevant to this study in different countries like USA, China, Singapore, this type of correlation study is a new opening in our country. (Armstrong et al., 1981, Xi et al., 2009) Therefore this study was primarily designed to focus on the residence of sensitivity as well as resistance of several antibiotic resistance genes from the source of hospital waste water and nearby community tap water. The results showed the presence of resistance in penicillin such as-ampicillin, cefixime, amoxiclav resistance in a 6 months laboratory observation of *Klebsiella*

pneumoniae. In addition to that the good news for an overpopulated country like ours where awareness of people and control of antibiotic uses are challenging, we did not find any resistance against carbapenem, imipenem or beta lactamase antibiotics.

Chapter 1

Introduction & Literature Review

1.1. Background

If we look back just a few months we can see the sudden outbreak of cholera in Dhaka city which was suspected to be spreading from the WASA supply line in Dhaka city. The highly affected areas in Dhaka are Dakshinkhan, Jatrabari trailing and followed by them the higher affected areas include Mirpur, Mohammadpur and Sabujbagh. (MELS et al., 2022). According to a doctor from ICDDR, B the number of patients with diarrhea was surprisingly shocking since the number was more than double compared to last year! (Breaches in Wasa Supply Lines Causing Cholera Outbreak in Dhaka? n.d.) Therefore, according to ICDDR, B it was a sudden outbreak where half of the cases were found to be caused by a heterotrophic bacterium known as vibrio cholerae while the other half is non-identified. Now, in the same interview of that news the operator of Dakshinkhan's Mollapara water pump put the explanation of this sudden incident by claiming that there was a renovating work ongoing for installing new supply lines and old ones were to be discarded or the supply to be stopped but in between this working system somehow sewage line got leaked or mixed up and caused the contamination and that was the probable reason for water contamination of that area. Now, well coming back to my study, this news and the real scenario inspired us to develop a hypothesis of something like maybe there is a correlation between hospital wastewater and nearby community water being contaminated with several pathogens like vibrio, salmonella, *E. coli* and *Klebsiella*. So, we focused on *Klebsiella pneumoniae* for our study. Since this type of research is a bit less common in our country so we tried to establish a protocol that will support our hypothesis and along with that we can monitor on the organisms whether they are getting more resistant or not since hospital waste is a huge carrier of different antibiotics, chemicals as well as a source of different microorganisms to harbor in that water and may be for some mistakes in supply system or the lacking of proper wastewater effluent treatment is a suspected reason to create another outbreak with another organism which we might be able to predict by monitoring them in a yearly observation. Now, there comes the question, why Klebsiella pneumoniae and no other organisms? Well, we microbiologists or biotechnologists or doctors or people related to scientific studies are mostly aware of the fact that *Klebsiella pneumoniae* is a highly emerging pathogen that causes pneumonia in general but it gets difficult to treat people with other existing diseases or admitted in hospitals or are immunocompromised with even carbapenem antibiotics which are highly designed to fight against multi drug resistant organisms infection and infect Klebsiella is unfortunately one of them. Moreover, not to mention, this organism is also responsible for causing urinary tract infection. (Martin & Bachman, 2018)

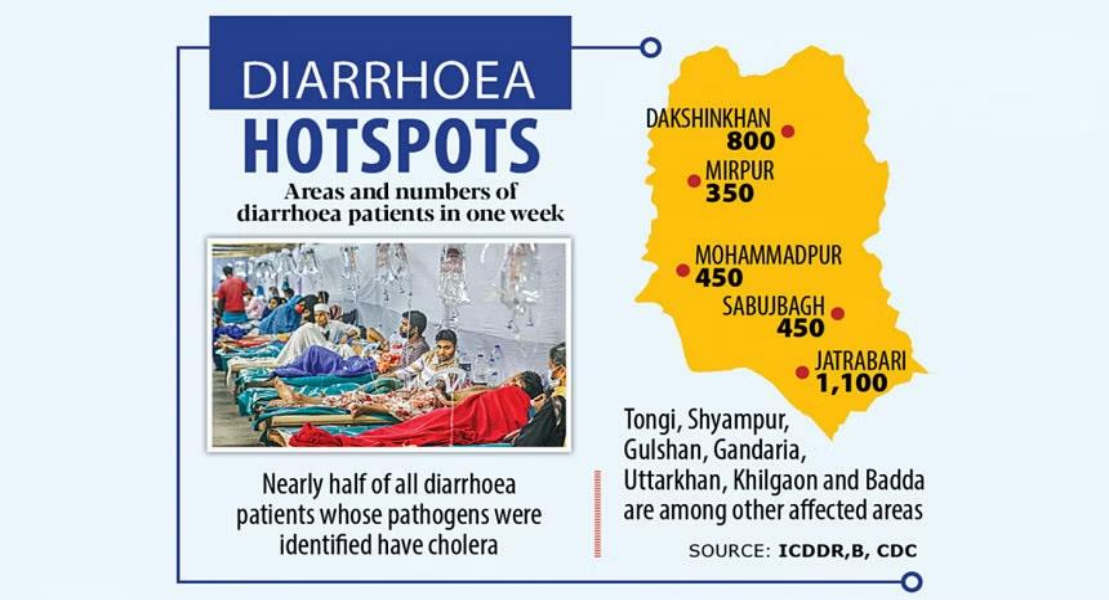


Figure 1.1

Source: ICDDR, B & The Daily Star.

1.2. Characteristics of *Klebsiella pneumoniae*:

It is a well-known gram-negative, capsule containing, immotile bacterium belonging to the group of enterobacterial. That being said, it represents the organism being capable of fermenting sugar in different biochemical tests. It is present in the environment and has been found to be linked to developing pneumonia in patients with diabetes, lung weakness including kidney disorder patients and other immunocompromised people. The bacterium is commonly found to be colonized in the oropharynx and the mucosa of gastrointestinal tract of humans as well as the urinary tract of mostly in patients with other physical problems and admitted to hospital. (Ashurst & Dawson, 2022) It is able to exhibit concerning pathogenicity along with antibiotic resistance which becomes difficult to treat with 1st and 2nd or even 3rd generation antibiotics once it gets inside the host successfully. Moreover, this bacterium alone is causing around 3% - 8% of all hospital pneumonia which is categorized as nosocomial infections and in present scenario it is considered throughout the world that it is responsible for causing hospital-acquired or nosocomial pneumonia (especially, patients under ventilation) as well as urinary tract infection with chronic kidney disease and people with other immunocompromised diseases (Ashurst & Dawson, 2022).

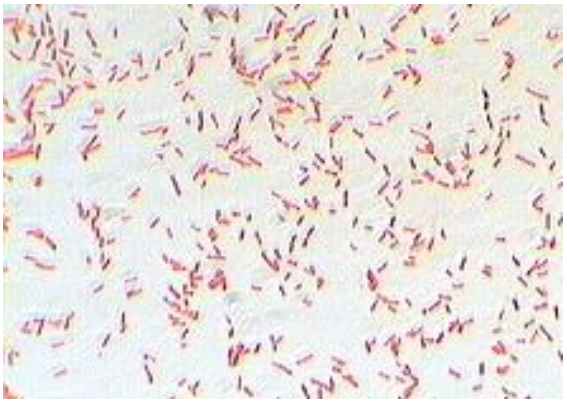


Figure 1.2: *K. Pneumoniae* gram stain
Image source: (complete profile, 2010)

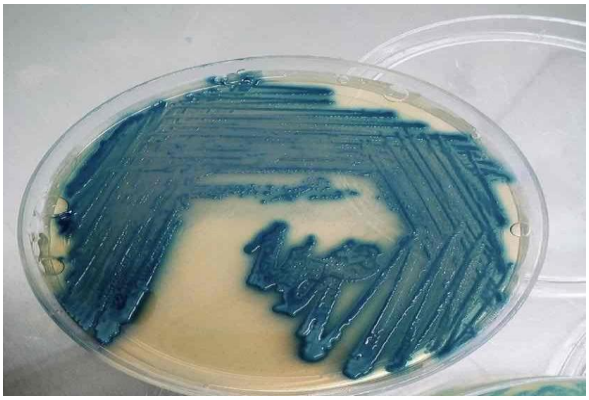


Figure 1.3: *K. Pneumoniae* on KPC agar
Image source: (Bagley & Seidler, 1978)

Figure 1.4: *K. Pneumoniae* on MacConkey agar
Source: (Bagley & Seidler, 1978)

1.3. Virulence Factors:

In order for a bacterium to infect its hosts, it requires a number of virulence factors that helps the bacterium to invade its host and cause infection where several virulence factors can be involved. Since most of the pathogens require virulence factors to be able to invade a host body along with its defense system to reproduce in there and for survival necessity of that organism. The infectious capabilities of *Klebsiella pneumoniae* which are known as virulence factors is contributed by a diverse set of conditions, any of which may result in infection or antibiotic resistance. Firstly, the capsule in the cell wall of the organism made of polysaccharide is one of the most crucial virulence factors for almost every gram-negative organism and it provides the bacteria with the ability to avoid phagocytosis and serum death by the host defensive mechanisms. Till now, 77 distinct forms of capsules have been identified and it has been found that any of the *Klebsiella* species that lack a capsule in its outer coat is typically less dangerous or virulent than the others having capsules

just like other infectious pathogens.(Deshpande et al., 2018) Secondly, the most potential virulence factor is the lipopolysaccharides that cover the outside layer and found in most of gram-negative bacteria which is identified usually by gram staining method under microscope and can be found on the bacterial cell wall. The detection of lipopolysaccharides triggers an inflammatory cascade involving cytokines, interferons, B cells, chemical mediators and other immune cells to overcome the invasion or other injury or infection in the host and has been identified as a primary contributor to the sequela meaning having a disease already in the host making the host more vulnerable and can lead the host to develop septic shock and sepsis. Thirdly, another factor that contributes to the virulence is the ability of the bacterium to fasten itself with the host cell receptors that allow it to attach and that is enabled by another hostility component called fimbriae. Well, so far these were the common virulence factors found in most pathogens but some are more advantageous compared to the others, which possess the siderophore. Siderophores are responsible for extracting iron from the host in order to facilitate the spread of the infecting bacterium and the target organism of our study *Klebsiella pneumoniae* happens to have it which makes it more infectious and dangerous to dig in more about it. (Luan et al., 2018)

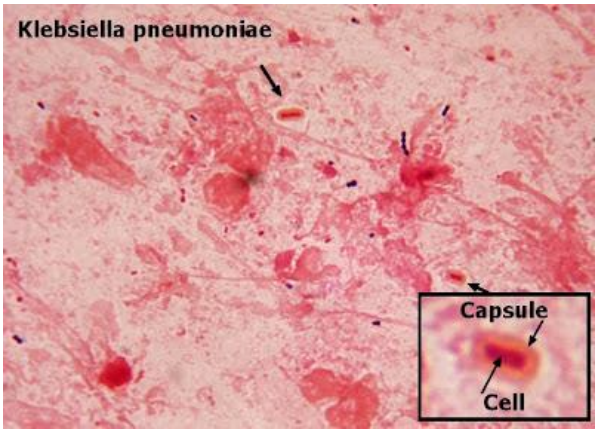


Figure 1.5: *K. Pneumoniae* capsule stain
Source: (complete profile, 2010)

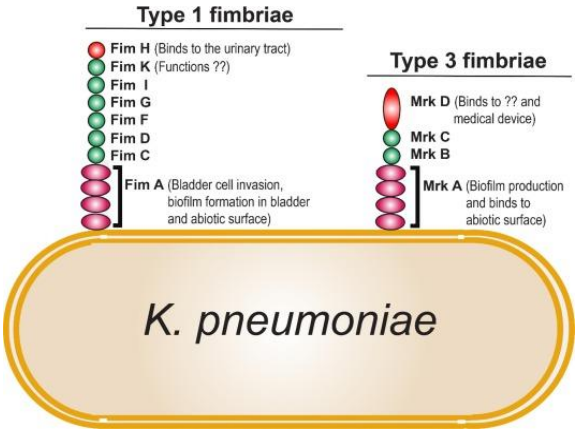


Figure 1.6: *K. Pneumoniae* fimbriae structure
(Govindarajan & Kandaswamy, 2022)

1.4. Etiology:

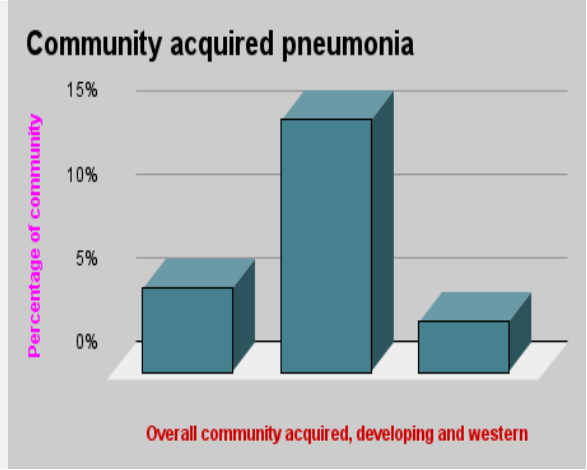
K. pneumoniae is primarily found in the human population and is transmitted from person to person through coughing, airways. In the general population, anywhere from 5 percent to 38 percent of people have this organism in their stools, and anywhere from 1 percent to 6 percent of people carry it in their nasopharynx. (Walter et al., 2018) The gastrointestinal tract of the patient and the hands of hospital staff members are the most common sources of infection in the hospital which may result in the spread of a nosocomial infection. On the other hand, studies have shown that people of Chinese origin and those who suffer from chronic alcoholism have significantly greater rates of colonization. The rate of *K. pneumoniae* carriers among hospitalized patients is

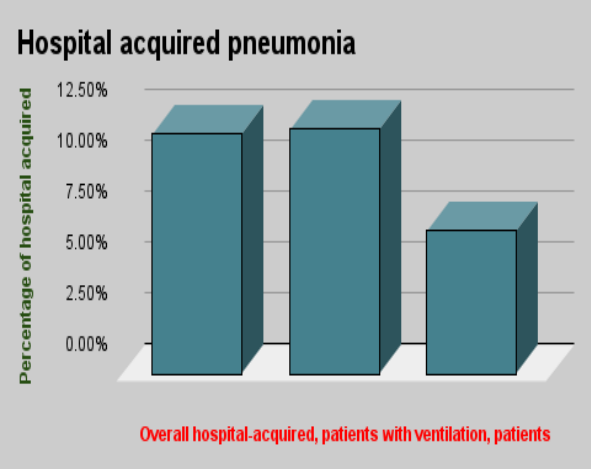
significantly greater than the rate discovered in the general population. (Walter et al., 2018) According to the findings of one study, carrier rates in the feces of hospitalized patients can reach as high as 77 percent and are connected to the total amount of antibiotics administered (Esposito et al., 2018).

1.5 Pneumonia:

Community-acquired pneumonia and hospital-acquired pneumonia are the two classifications that can be used to describe pneumonia that is caused by *K. pneumoniae*. Although a diagnosis of community-acquired pneumonia is very prevalent, this type of infection by *K. pneumoniae* is not very common at all. *Klebsiella pneumoniae* will often only affect the upper lobes of the lungs, but it is not impossible for it to spread to the lower lobes as well. Crepitation, bronchial breathing, and increased vocal resonance are typical examples of unilateral symptoms of consolidation that can be found during the test for the most part and in the upper lobe as well. (Cerwenka, 2010)
K. pneumoniae has symptoms that mirror those of common colds and other forms of pneumonia that is acquired by community. Patients usually experience the symptoms such as coughing, fever, chest pain (pleuritic) and difficulty breathing. The sputum that is produced by Streptococcus pneumoniae and the sputum that is produced by *K. pneumoniae* infections are very different from one another. Those infected with S. pneumoniae typically cough up a sputum that has a "blood-tinged" or "rust-colored" tint, whereas those infected with *K. pneumoniae* cough up a phlegm that looks like "currant jelly." Infection with *K. pneumoniae* causes severe tissue necrosis and inflammation, which is why these conditions prevail. (Mandell et al., 2007)
It is roughly calculated that about 3 percent to 5 percent of total cases of pneumonia that belongs to community-acquired classification are responsible for getting an infection caused due to exposure to *K. pneumoniae*. On the other hand, a contrast estimation is found for the countries that are under developing like Africa, the infection caused by *K. pneumoniae* is approximately estimated to be around 15 percent of most cases found recorded as pneumonia. Again, for the western communities, the estimation is about 3 percent to 5 percent for each of the pneumonia cases in the class of community-acquired infection. In the grand scheme of things, roughly 11.8 percent of total cases of pneumonia record belongs to the hospital-acquired category in the world that can be attributed to *K. pneumoniae*. In addition to that *K. pneumoniae* is responsible for 8 percent to 12 percent of cases of pneumonia in patients who are being treated with a ventilator. However, this bacterium is only responsible for 7 percent of pneumonia cases in patients who are not being treated with a ventilator. Patients who have both alcoholism and septicemia have a mortality rate that varies from 50 percent to 100 percent. (Moemen & Masallat, 2017)

Diagram 1: Community acquired pneumonia vs Hospital acquired pneumonia





1.6. Other concerns apart from Pneumonia:

Klebsiella is responsible for a wide variety of infections that may be found in communities, long-term care institutions, and hospitals all over the world. These infections can affect the lungs, abdominal cavity, urinary system, circumcisional sites as well as soft tissues. They can also cause bacteremia (Peermohamed & Kogilwaimath, 2018). Since this pathogenic bacterium can be colonized as part of the common and regular flora of the skin, mouth and intestines, there is high chance that it might become opportunistic whenever the host immune is down or the host immune is busy fighting against other target pathogens. Moreover, patients of sepsis are easily getting exposed to this bacterium and as a result it has now become the third most often identified organism in the cultures of blood tested from this type of patients. A new hypervirulent variation of *K. pneumoniae* has been found, and it is becoming a public health problem as a result of its ability to cause serious and lethal infections and may arise untreatable due to a strain of MDR of this bacterium. **These diseases include meningitis mostly in infants but also in elderly people, pyogenic liver abscesses, endophthalmitis and UTI.** (CRISTEA et al., 2017) The prevalence of *Klebsiella pneumoniae* uropathogens in chronic kidney disease (CKD) is studied in a research which showed that between July 1 to December 31 of 2010, in the Department of Nephrology 357 patients were admitted to hospital due to the severe situation, surprisingly among them 37 cases had UTIs (10.36%) along with 12 cases of CKD (32.43%). From that same study it is recorded that UTI caused by K. pneumoniae, the cases are 10 for male (27.03%) which is a bit concerning since UTI is commonly an issue of suffering for women and 2cases are for female (5.40%) patients. This research was conducted between July 1 and December 31, 2010. (CRISTEA et al., 2017)

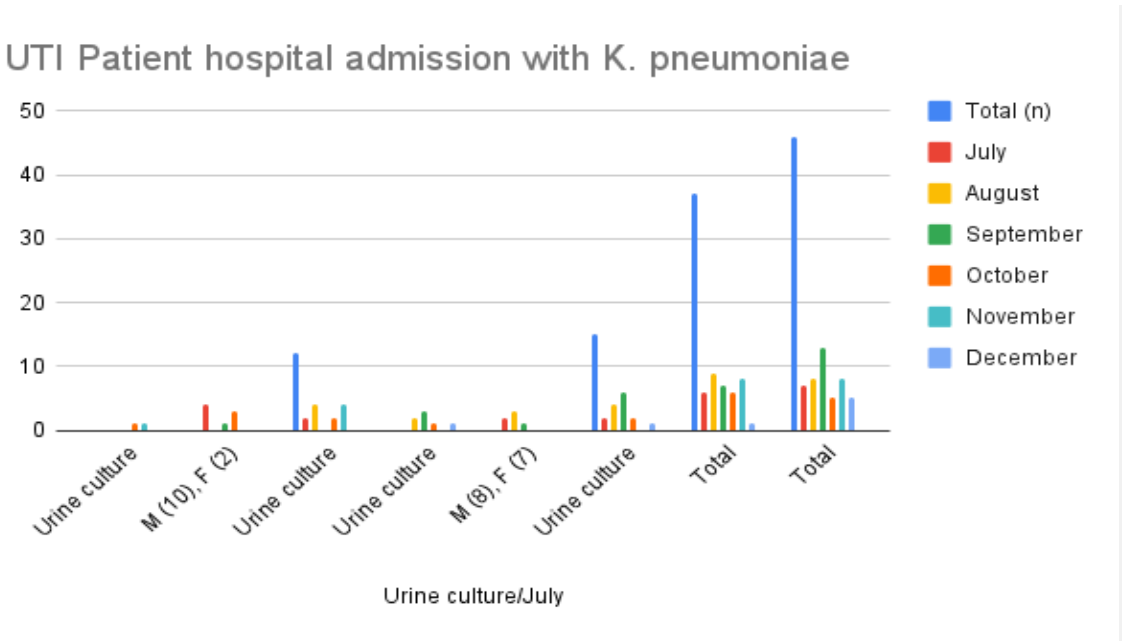


Diagram 2: *Klebsiella pneumoniae* relation with UTI and CKD patients

Source: (CRISTEA et al., 2017)

1.7. Why the situation is a matter of concern:

Antibiotic resistance has been identified as an all-inclusive concern in terms of this present-day medicine as a result of extensive widespread use of antibiotics more than seven decades to treat infectious diseases and infections which is truly a long time and we must consider ourselves lucky enough that we are still left with antibiotics that are in working condition but soon we might lose them as well because of our unawareness and careless actions of not only toward the drugs but also to the environment we live in . (The Review on Antimicrobial Resistance, O'Neill 2016). Multidrug-resistant (MDR) and extremely drug-resistant (XDR) organisms categorized as most untreatable pathogens also classified to the members of Enterobacteriaceae group are ordinary habitants of our natural microbiome, hence the substantial increase in the occurrence of illnesses caused by these pathogens is very concerning. In addition, infections brought on by these strains tend to have high death rates, require extensive hospital stays, and incur considerable expenses. (Giske et al.2008). The development of resistance to antibiotics is a multifaceted and complicated activity. (Watkins and Bonomo 2016) Yet, when viewed from the standpoint of bacteria, it depicts the process of evolution in action, which is concurrent with the ongoing exposure to antibiotics (Davies and Davies 2010). This is because antibiotics create an environment where selection pressure leads to the creation of multiple genetic pathways. Because of this ongoing evolution over the course of many years, MDR and XDR Enterobacteriaceae strains have emerged. These strains of Enterobacteriaceae are resistant to almost all antibiotics that are currently in use, leaving no

viable treatment choices. The possibility of the global dissemination of these extremely drug-resistant bacteria has emerged as a recognized global danger. (Hersh et al.2012). Well, now we know that this organism possesses a convenient survival mechanism for itself which is causing the burning issue for human health concerns. So, looking after several scientific studies it has been found and also established by other scientific researches that this organism contains resistance genes such as β-lactam resistance, Broad-spectrum and (ESBL) extended-spectrum β-lactamases, Plasmid-mediated AmpC, Aminoglycoside, Quinolone, Polymyxin, Tigecycline, Plasmids associated with carbapenemase genes. (Navon-Venezia et al., 2017) Therefore, all these extra abilities led this organism to be a matter of concern and so in this study the focus was kept on the growth, availability and antibiotic susceptibility test for any resistance against any antibiotic. Therefore, to justify the hypothesis of this study all this information is enough to work on this research.

1.8. Establishing the hypothesis with *Klebsiella pneumoniae* (Objective and Justification):

Firstly, to begin with we already have an incident happened not to past rather too close in terms of time that makes us think that the water which is the major portion of our body and we named it life after all its contributions to our life is safe or not. It does not really matter whether it's a drinking water or a sewage water because in our country the waste water gets mixed with common natural water resources like ponds, rivers, lakes and ocean. Everything is interconnected and each action we take it affects our environment as well as the surrounding living beings including the complete ecosystem. Secondly, sewage water contains not only wastes we produce but also chemicals, antibiotics, so, it serves not only as a reservoir of nutrition but also to have advantage of getting the microorganisms gene alteration by different mechanisms such as transduction, transformation and conjugation known as basic gene modification mechanisms. Thirdly, the waste management in allover Dhaka city including industrial waste, hospital discharge and residential waste are not developed by following a proper planning of which is a part of urban planning. Therefore, the mix up or leakage is nothing surprising at all! Finally, the supply water for drinking purpose in residential areas can get exposed to any kind of waste water and the result could end up in another outbreak like cholera, diarrhea, UTI, typhoid or other infections we have not think of yet due to all those invisible microscopic beings thriving in those waste water and getting opportunities to contaminate the community water! So, to validate our thesis research hypothesis, all these reasons are good enough to have the chances to monitor as well as observe antibiotic sensitivity and resistance among different organisms which in our case is *Klebsiella pneumoniae*.

1.9: Relativity & Similarity with other studies:

So, in our study we mainly focused on hospital waste water and finding out correlation of contamination between community water supply. In the result and discussion part all our findings have been showed and analyzed. Now, to support this study and its relativity an article where a

research has been done on District hospitals and local Mother and Child Welfare center facilities in Bangladesh. In that study it has been shown that none of the healthcare facilities maintain the appropriate standard waste management. Even in that study it has been shown that different units of hospitals for example, pathology unit, pediatric unit, operation theater and emergency room wastes are treated by only segregation before releasing them through the liquid sewage line. In district hospitals the highest waste management was shown only more than 60% for OT whereas in the Mother and Child Welfare center facilities the rate of waste management was 0% for pathology, pediatric and emergency unit and the waste treatment for OT could not even pass more than 25%! (Sujon et al., 2022) These are all kind of an interconnection and serve as a great source of spreading contamination. As a consequence, the whole ecosystem balance of human>>animals>>microorganisms>>plants and overall the environment gets exposed to a huge source of drugs, chemicals, microorganisms, infectious ingredients and so on. Therefore, not to mention that leakage in WASA supply line in community is a very common incident for the people of Dhaka and the supply lines are sometimes just open, old equipment which are in bad condition, lacking of planned system and management, mainly these lines are connected under the soil. So, here we can find a probable possibility that somehow there is a chance that the organisms can spread from any kind of source where we focused on hospital waste water and can contaminate the community water. Similarly, in another study it has been found that from environmental water sample the presence of *Klebsiella pneumoniae* is a concerning matter of fact. Because this organism is worldwide known as one of the major threats that can cause serious diseases like meningitis, epidural abscess, endophthalmitis, bacteremia, necrotizing of soft tissue, bone and most commonly nosocomial pneumonia and UTI. The major concerning fact is that it contains extended spectrum beta lactamase as well as carbapenemase which make this organism multi drug resistant and difficult to treat critical infections. Again, this organism contains MDR loci along with plasmid that can easily spread or share genes through horizontal gene transfer such as- Transformation, Transduction and Conjugation. A strain of this bacterium found to carry five different plasmids which make it more defensive in terms of evading host defense mechanism for example it contains mechanism against human complementary system which is an essential part of human immune system. (Martin & Bachman, 2018)

Also, the resistance pattern of this organism is a matter of concern if this study purpose is compared and related with other studies where the focus was only *Klebsiella pneumoniae*, its virulence abilities and increasing resistance towards multiple drugs (Habeeb et al., 2007). Two charts are attached below for the antimicrobial resistance pattern of this organism-

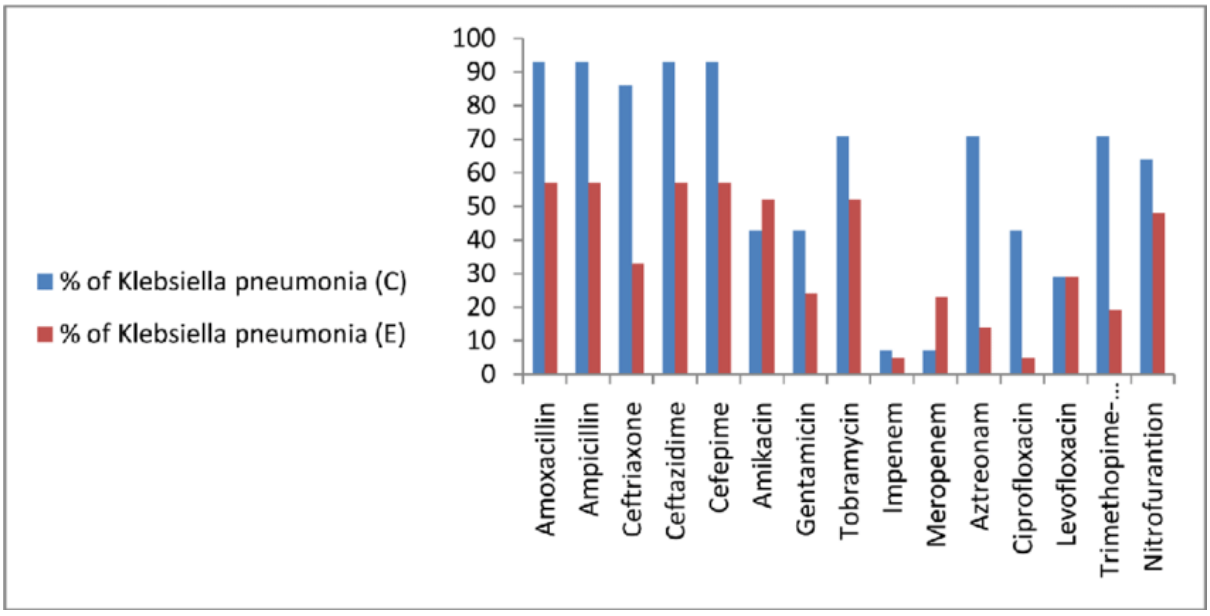


Diagram 3: *Klebsiella pneumoniae* isolation from clinical & environmental sample and its resistance pattern for different antibiotics

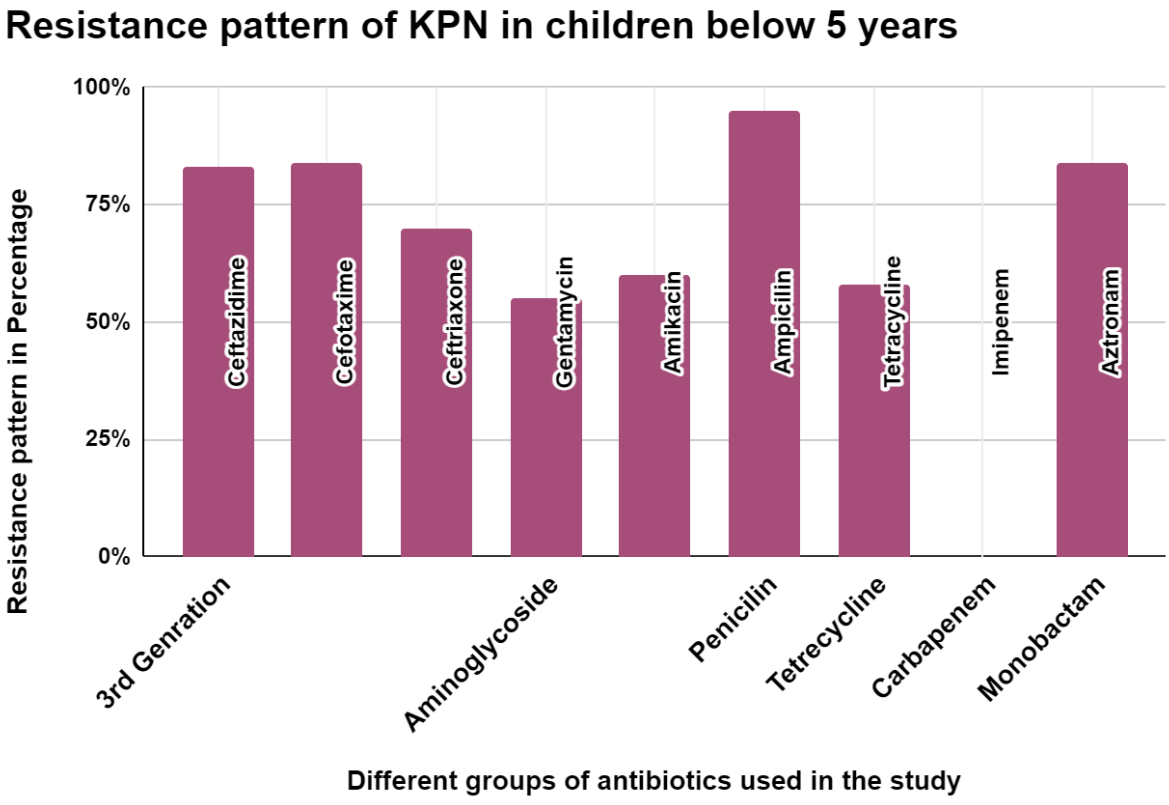


Diagram 4: Resistance pattern in *Klebsiella pneumoniae* isolated from patients below 5 years
Source: (Habeeb et al., 2007)

Chapter 2

Methods and Materials

2.1. Work flow of our study:

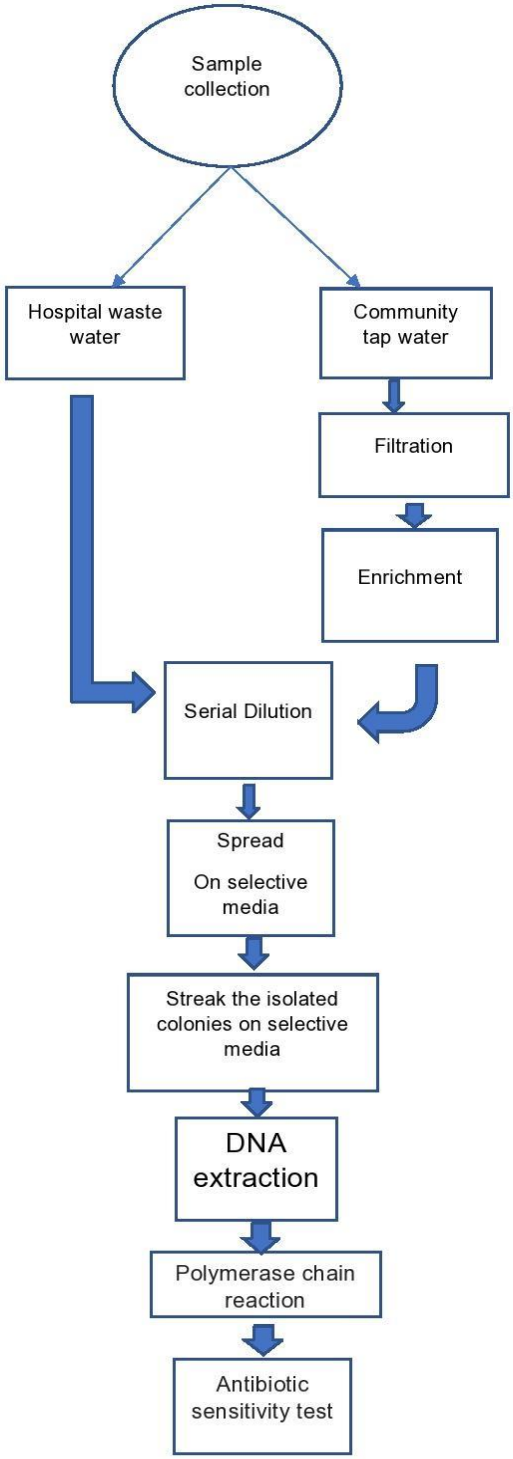


Figure 2.1

2.2. Media, Solutions, and Reagents that has been used:

The media, reagents, and solutions utilized in this thesis were obtained in reagent grade quality and were used without any additional purification.

BPW:	Buffered peptone water is used as non-selective pre-enrichment broth to increase the bacterial concentration of community water.
KPC:	Klebsiella pneumoniae carbapenemase media is used as a selecting media to grow Gram-negative bacilli.
ESBL:	Extended Spectrum Beta-Lactamase media is used as another selective media to grow Gram-negative bacilli.
MHA:	Mueller-Hinton agar is not a selective media. It is a nonselective agar which is commonly used solid growth medium in microbiology for the cultivation and antimicrobial sensitivity testing of a wide range of bacterial species. We used MHA to perform antimicrobial sensitivity test.
NA:	Nutrient agar is a versatile growth medium that is suitable for cultivating a broad range of non-filamentous microorganisms. It has been used to subculture the selected organism before antimicrobial sensitivity test and stock them.
TE buffer:	Tris-EDTA buffer is used in extraction of DNA.TE buffer helps to maintain the stability of DNA during extraction and storage.
TBE Buffer:	TBE or Tris-Borate-EDTA buffer is a commonly used buffer in gel electrophoresis for separating and analyzing nucleic acids, such as DNA and RNA.
EtBr:	Ethidium bromide (EtBr) is a commonly used fluorescent dye in gel electrophoresis to visualize DNA or RNA bands. When added to the gel or running buffer, EtBr intercalates between the base pairs of the DNA or RNA molecules, causing them to fluoresce under UV light.

2.3. Media preparation:

2.3.1. BPW:

- We used buffered peptone water as enrichment media.
- To prepare 1000 ml of BPW, we need tryptone 10g, NaCl 5g, 1.5g potassium dihydrogen phosphate and 3.5g di-Sodium hydrogen phosphate to add in 1000 ml of distilled water.
- Later It was boiled to dissolve, and autoclaved for 15 minutes at 121° C to sterile. (Oxoid - Product Detail, n.d.)

2.3.2. Hichrome KPC:

- For the selected pathogen in the study, Hicrome KPC media was utilized for culturing.
- The standard amount of KPC media, 16.50 grams in 500 ml of distilled water, was used as a reference for preparation, and the required amount was prepared accordingly.
- The media was heated until it was fully dissolved and then autoclaved at 121℃ for 15 minutes.
- Subsequently, the culture media was poured into sterile Petri dishes of varying sizes as needed and left to solidify before being stored in a refrigerator for future use.

2.3.3. Hichrome ESBL:

- HiChrome ESBL media was utilized to cultivate the specific pathogen for investigation.
- The accepted method of ESBL preparation involved dissolving 40 grams in 1000 ml of distilled water, which was used as a benchmark to determine the required quantity.
- After boiling, the solution was autoclaved at 121° C for 15 minutes, and subsequently, poured into sterile Petri dishes of varying sizes as needed.
- Once the agar had solidified, it was refrigerated for later use.

2.3.4. NA:

- For this experiment, Nutrient Agar (NA) was employed to subculture the chosen pathogen.
- The established formula for NA called for 28.0g to be dissolved in 1000 ml of distilled water, which was adopted as a reference to determine the appropriate amount.
- The solution was then boiled to ensure proper dissolution and autoclaved at 121° C for 15 minutes.
- It was subsequently poured into sterile petri dishes of various sizes as required and allowed to solidify before being stored in the refrigerator for future use.

2.3.5. MHA:

- MHA agar is a nondifferential and also nonselective medium used in this study.
- To prepare the MHA agar, 38.0 g was dissolved in 1000 ml of distilled water and used as a benchmark to determine the necessary quantity.
- The solution was then boiled to ensure proper dissolution and autoclaved at 121° C for 15 minutes.
- Subsequently, it was poured into sterile, large-sized Petri dishes and allowed to solidify before being stored in the refrigerator for later use.

2.3.6. T1N1:

- We used this agar for stocking bacteria for further research purposes.
- It is also used for decimal dilution & preparation of specimens for the purpose of microbial tests.
- For 100 ml of T1N1 agar, 1 g of tryptone, 1 g of NaCl, and 2 g of agar were needed to add in 100 ml of distilled water.
- Later It was boiled to dissolve, poured in sterile vials.

2.3.7. Soft agar:

- We used this agar for stocking bacteria for further research purposes.
- For 100 ml of soft agar, 0.8 g of nutrient broth, 0.5 g of Nacl, 0.8 g of agar powder were needed
- 100 ml of distilled were needed.
- Then, it was mixed thoroughly and boiled to dissolve properly and needed to autoclave.
- Then it was poured into sterile vials.

2.3.8. Preparation of physiological saline:

- For the antibiotic susceptibility tests, bacterial dilution was performed using physiological saline.
- To prepare the saline solution, 0.9 g of Sodium Chloride (NaCl) was mixed with 100 ml of distilled water in a clean conical flask to create 100 ml of physiological saline.
- The solution was then transferred into a 15 ml test tube with a volume of approximately 5 to 6 ml and autoclaved for later use.

2.4. Procedure of ample collection:

We collected water sample from the hospital effluents and hospital-adjacent community. The waste water from the selected hospitals and tap water from the selected hospital-adjacent community with specific distance which is within the range of 300 meter from the surrounding area of selected hospitals from which samples were collected in containers. Then carefully bring those in the laboratory to perform our study.

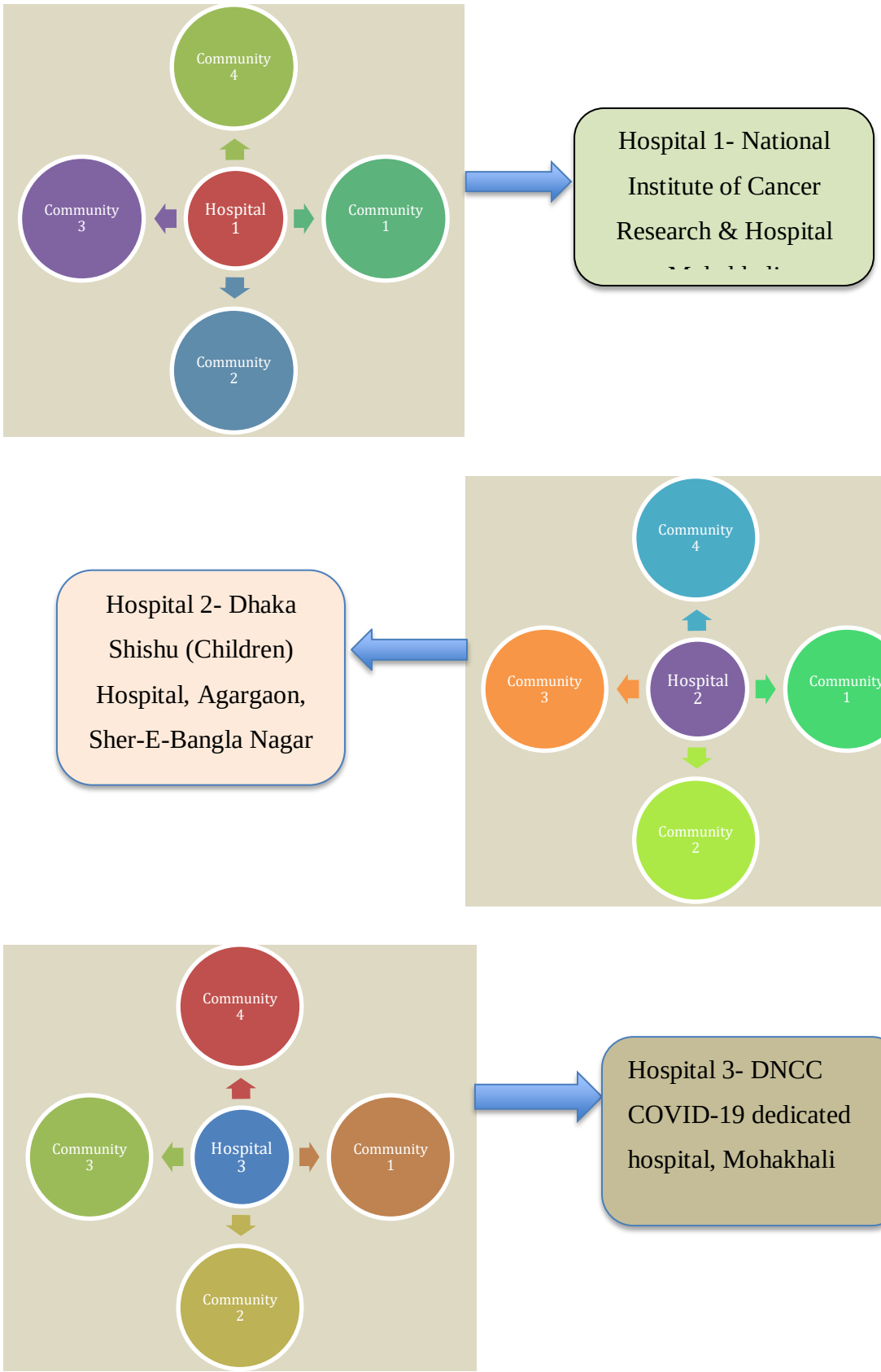


Diagram 5: An illustration of 3 different hospitals and their adjacent 4 community sources

Location and distances of communities' residences from hospitals in google map-

Figure 2.2: Map of the DNCC hospital effluents and hospital-adjacent communities

2.5. Handling the sample before culture:

Hospital samples are directly used for serial dilution but the community tap water is filtered with 0.45µm membrane filtration. After that, we transfer the membrane filter to 10ml of sterile Buffer Peptone water and incubate at 37°C for 4hrs. After incubation we use the BPW for serial dilution.

Figure 2.3: Collected hospital and community samples

2.6. Serial dilution:

Serial dilution is a technique that is used in laboratory to prepare a series of solutions with minimize the concentrations of a particular substance. It involves diluting a concentrated solution (known as the stock solution) with a solvent, such as water or buffer, to create a series of solutions with lower concentrations. Each subsequent solution in the series is made by taking a small volume of the previous solution and diluting it further with solvent.
We serial dilute both hospital(direct) and community (incubated in BPW) samples in saline water. After that, we used spread plate technique to grow bacteria. (Serial Dilution | Science Primer, n.d.)

Figure 2.4: Serial dilution

2.7. Spread plating:

The spread plate technique is a microbiological method used to isolate and quantify bacterial colonies from a mixed population.

- Firstly, we prepare the appropriate growth media which is HiChrome KPC media (without supplement) or HiChrome ESBL (without supplement) for the bacteria of interest, and sterilize it by autoclaving.
- After that, by using a sterile pipette, we place 10 mL of the diluted bacterial culture onto the center of a sterile Petri dish. We used dilution factor between 10^-2 to 10^-8.
- Then, using a sterile spreading tool, we spread the bacterial culture evenly over the surface of the agar.
- Allowed the plates to dry for a few minutes, then invert them and incubate them in at 37°C, 24hrs.
- After we done with the incubation, we counted the colonies that have formed on the surface of the agar. The number of colonies will depend on the original concentration of the bacterial culture and the dilution factor used.
- We selected the isolate colonies to use streak plate technique to obtain more specific discrete colonies. (MN Editors, 2022)

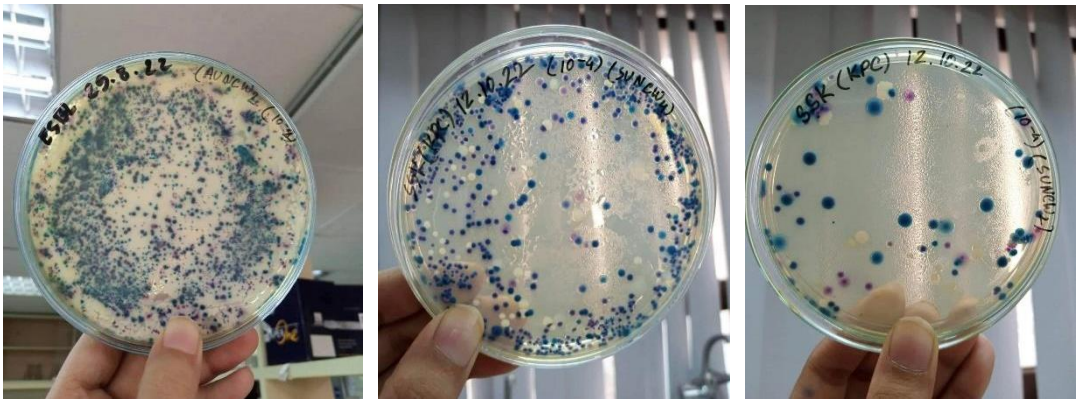


Figure 2.5: Spread plate on KPC and ESBL media

2.8. Streaking the selected isolates:

The streak plate technique is a microbiological method used to isolate and purify individual bacterial colonies from a mixed population. We select single isolated colonies for streaking on selective media such as HiChrome KPC media plates under an aseptic condition in a laminar airflow cabinet. After that, culture plates were incubated at 37° C for 24 hours aerobically. Here, HiChrome KPC agar was used for gram-negative bacilli (*Klebsiella* spp). Also, Gram Staining was performed for gram-positive and gram-negative bacteria, and visual observation of bacterial

colonies was done by microscopic examination for morphology, color, and shape. (Bacteria Streaking Methods and Isolation Techniques, n.d.)

- Firstly, we prepare the appropriate growth media which is HiChrome KPC media (without supplement) or HiChrome ESBL (without supplement) for the bacteria of interest, and sterilize it by autoclaving.
- Next, take a small amount of the mixed bacterial culture and streak it onto one quadrant of the petri dishes agar surface using a sterile loop.
- Then, we flame sterilize the loop by passing it through a flame until it glows red. Allow it to cool for a few seconds before proceeding.
- Then, from the initial streak, make a series of "zig-zag" streaks onto the next quadrant of the agar surface, spreading the bacteria over a larger area.
- Repeat step 3 to sterilize the loop again.
- From the second streak, make another series of "zig-zag" streaks onto the third quadrant of the agar surface, diluting the bacteria further and repeat step 3 to sterilize the loop again.
- From the third streak, make a final series of "zig-zag" streaks onto the final quadrant of the agar surface, isolating individual bacterial colonies.
- After that, we allowed the plate to dry for a few minutes, then invert it and incubate it 37°C for 24hrs.
- After incubation, observe the colonies that have formed on the surface of the agar. Select a single, well-isolated colony for further analysis like sub culturing, gram-stain or DNA extraction. (Bacteria Streaking Methods and Isolation Techniques, n.d.)

Figure 2.6: streak plate on KPC media

2.9. Gram stain:

- First, we make the smear and five milliliters of freshly produced saline were used.
- The slides were covered with a loopful of saline and then added together with a small amount of the isolate which has been heated to fix it.
- After that, crystal violet was added and left on the smear for between 30 and 1 minute.
- Next, the area where we smeared was rinsed with distilled water.
- Finally, Gram's iodine, a mordant was applied. left on the smear and wait for a minute.
- Following that, rinse with distilled water.
- We then added ethanol or acetone and wait for a further 15 seconds.
- Safranin is then included.
- Slides were prepared to examine under a microscope after being air dried.
- Under a microscope, gram-positive bacteria can be seen as purple colored cells, whereas gram-negative bacteria can be seen as pink or red colored cells.

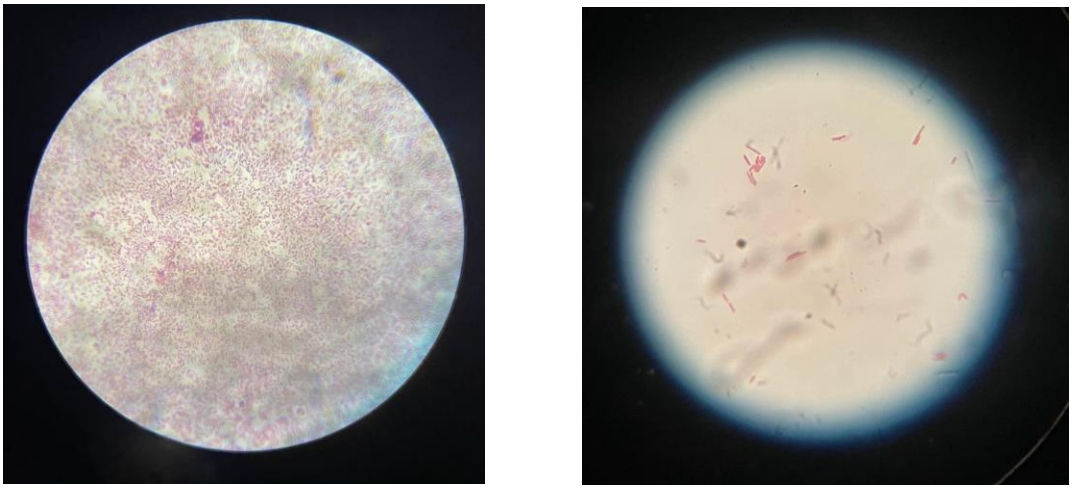


Figure 2.7: Gram-negative rod-shaped bacilli

2.10. DNA Extraction:

DNA is isolated from cells through a process called DNA extraction that involves rupturing the nuclear membrane and cell wall. The cell must first be ruptured in order to release the nucleus, which must then be opened in order to release the DNA. Once the DNA has been isolated, it needs to be kept apart from other biological components and shielded from the DNA-decaying enzyme DNase. DNA can be extracted using a variety of procedures, including enzymatic, mechanical,

and boiling ones. Based on the particular conditions and demands of the experiment, the best method should be selected.
DNA extraction was done using TE buffer. For a number of reasons, TE buffer (Tris-EDTA buffer) is frequently employed in DNA extraction and purification techniques.

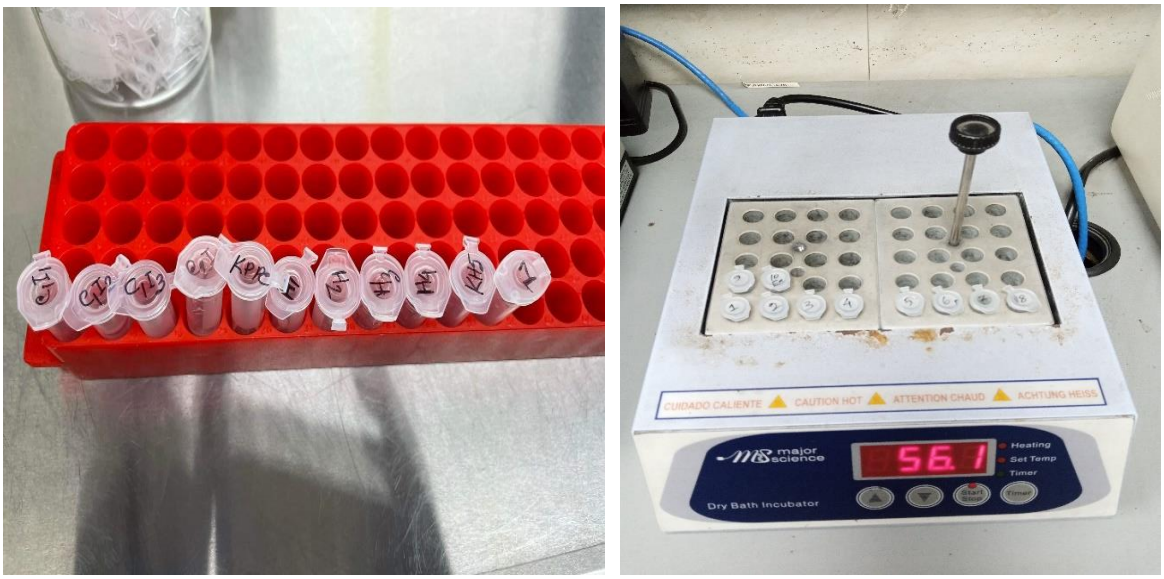


Figure 2.8: Extraction of DNA in MCT & Dry heat boiling machine

DNA stability: During DNA extraction and storage, TE buffer aids in maintaining DNA integrity. The Tris component of TE buffer serves to keep the solution's pH stable, preventing acid hydrolysis from degrading DNA. Metal ions that have the potential to cause DNA destruction are chelated by the EDTA component. (Chauhan, 2021)

Dissolving DNA: Additionally, DNA pellets that may develop during the extraction and purification processes are dissolved using TE buffer. The buffer aids in solubilizing the DNA and guards against its undesired or complete denaturation or damage.

Enzymatic reactions: TE buffer is often used as a reaction buffer for enzymatic reactions that involve DNA, such as PCR (polymerase chain reaction) and restriction enzyme digestion. The buffer helps to maintain the pH and ionic strength of the reaction mixture, which is critical for the proper functioning of the enzymes. (Chauhan, 2021)

We used boiling method to extract DNA.

- First, we used 1.5 ml microcentrifuge tubes to hold 150 ml of TE buffer.
- Utilizing the loop inoculation procedure, inoculate the discrete, isolated colonies in the TE buffer.

- After the resuspended cells have been boiled for 15 minutes at 100 degrees Celsius to lyse them and release their DNA, pellet the cells by centrifuging them at 14,000 revolutions per minute for 5 minutes at room temperature.
- Discard the pellet and keep the supernatant at a temperature of -20 C. (Ahmed & Dablool, 2017)

2.11. PCR or Polymerase Chain Reaction:

By maintaining a heat cycle, the targeted DNA sequence can be rapidly multiplied into millions of copies using the DNA amplification technique known as PCR. DNA polymerase is employed to create 12 new copies of the target sequence, while primers are oligonucleotide sequences that provide the PCR reaction specificity. The PCR reaction is supplemented with all the elements required to synthesize fresh copies of the targeted area. (De Pietro Crt, 2022)
A specific DNA fragment can be amplified in a lab setting using the PCR (Polymerase Chain Reaction) method. The general steps to execute PCR are as follows:

DNA template preparation: The first step is to prepare the DNA template that will be amplified by PCR. This may involve extracting DNA from a sample, purifying the DNA, or using a pre-existing DNA sample.

Primer design: Designing the primers that match the target organism DNA sequence at the two ends of the target region that provides the sequence to be synthesized and amplified inside the PCR machine.

PCR preparation: Preparing the reagents and extracted DNAs for PCR reaction is conducted in a sterile tube known as micro centrifuge tube (MCT) or Eppendorf and PCR tubes, which includes the DNA template, forward and reverse primers, a DNA polymerase enzyme, dNTPs (deoxyribonucleotide triphosphates) included in the PCR master mix and nuclease free water.

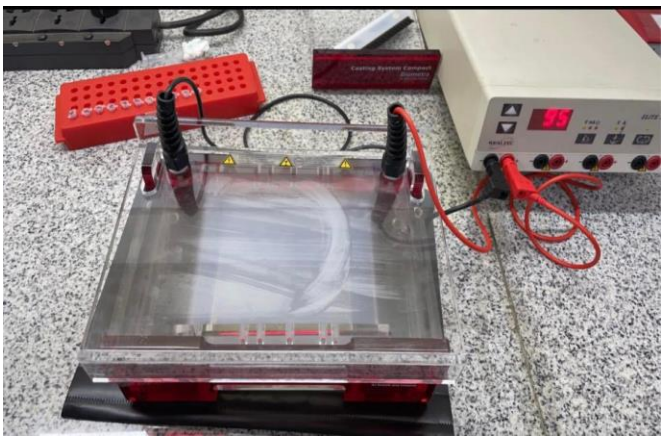
PCR cycling: In a thermal cycler, the PCR reaction mixture goes through a number of temperature cycling phases. Denaturation of the double-stranded DNA occurs between 94 and 98 degrees Celsius, annealing of the primers to the template DNA occurs between 50 and 65 degrees Celsius, and primer extension by the DNA polymerase enzyme occurs at 72 degrees Celsius. Normally, these processes are performed 25 to 40 times. (De Pietro Crt, 2022)

Figure 2.9: PCR products on PCR tubes and insertion of PCR products on PCR machine

2.12. Gel Electrophoresis:

Gel electrophoresis is used for separating molecules like DNA or RNA or proteins based on their size and charge. During the process, an electrical current is applied to the gel, causing the molecules to migrate towards the positive electrode and become separated based on their size. Typically, larger molecules move more slowly through the gel than smaller ones. To determine the size of the separated molecules, a DNA ladder with known sizes is used as a reference. (Steward, 2023)

We used gel run after PCR to confirm the band size of DNA. The band size of the primer we used was 130. To perform the gel electrophoresis, we used TBE buffer. Usually, we can use TAE or TBE buffer to perform gel electrophoresis. But we used TBE buffer because of our desired DNA size. In terms of composition, the primary distinction between TBE and TAE lies in their chemical makeup. TBE is a buffer that is made with EDTA, Tris, and boric acid and on the other hand TAE is composed with EDTA, Tris base and glacial acetic acid. When working with larger DNA fragments or for cloning, TAE is the method of choice, but TBE is suitable for achieving high resolution for short DNA fragment.



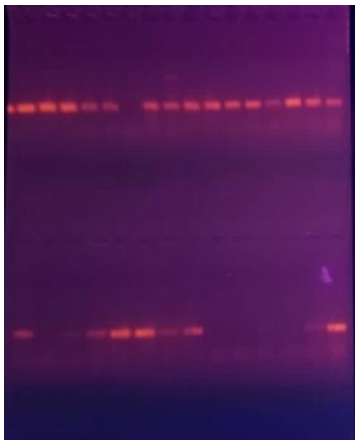


Figure 2.10: PCR products before loading on gel, Gel electrophoresis machine & PCR result under UV light

2.13. Antibiotic Sensitivity Testing:

To determine the best treatment for an infection, an antibiotic sensitivity or susceptibility test is conducted to identify the specific antibiotic and the recommended dosage. This test is essential in cases where the infection is resistant to antibiotics, indicating that antibiotics may not be effective against specific bacteria, making it more challenging to treat bacterial infections and illnesses. In this present study, the Kirby-Bauer method, a commonly used disk diffusion method, was utilized for antibiotic sensitivity testing. To ensure the accuracy of sensitivity patterns, all samples were tested twice. The following is the work procedure:
To prepare the inoculum, a fresh nutrient media culture plate was chosen, and a colony from the plate was collected using a sterile loop. After that inoculated the colony using sterile loop in the test tube that is containing sterile saline solution. Finally, the test tube was properly vortexed to achieve a homogeneous suspension.

To carry out inoculation on Muller Hinton Agar (MHA) plates, non-selective and non-differential Muller Hinton agar plates were prepared. The surface of the plate was then covered with a representative bacterial suspension prepared in physiological saline, using a sterile

cotton swab. The bacterial suspension was compared with the McFarland standard 0.5 solutions, which indicates a density of 1.5×10-8 CFU per ml. The cotton swab was pressed against the test tube wall to eliminate extra fluid before preparing the grass. The plate was then repeatedly swiped with the swab from various angles to ensure a uniform distribution of the bacterial suspension.

Placing the antibiotic disks on MHA plates: In this study, antibiotic discs were placed on solidified agar plates that a lawn of bacteria is created initially, using sterile forceps. A total of 11 antibiotics were used, including 10 for Gram-negative bacteria and 9 for Gram-positive bacteria. Once the discs were in place, the plates were covered with their lids and left upright before being incubated at 37°C for 24 hours.

Measuring zone: The diameter of the zone of inhibition was measured in millimeters using a ruler after the 24-hour incubation period. The zone was clearly apparent when the ruler was placed up against the plate's back. The antibacterial pattern was identified based on the presence or absence of a clear zone surrounding the antibiotic disc. In general, an antibiotic susceptibility test can yield one of three outcomes. The zone measurement was then interpreted using standard guideline of CLSI.

Such as:
● Susceptible or Sensitive (S) - This indicates that the organism can be effectively treated with the antibiotic at the recommended level, as evidenced by the moderate-sized zone around the antibiotic disc.
● Intermediate (I)-This statement pertains to organisms that have a moderate level of susceptibility to an antibiotic.
● Resistant (R)- This implies that there won't be a distinct zone around the antibiotic disc, indicating that the organism won't exhibit any reaction to the particular antibiotic.
The Clinical and Laboratory Standards Institute (CLSI) guidelines have classified antibiotic resistance patterns into MDR, XDR, and PDR.
● MDR (Multidrug-resistant) Bacteria - When a single bacterium is resistant to at least three classes of antibiotics then it is known as MDR.
● XDR (Extensively drug-resistant) Bacteria- When a bacterium remains sensitive to one or two antibiotics then it is known as XDR
● PDR (Pan Drug-resistant) Bacteria - These bacteria are non-susceptible to any drugs which are clinically proven. PDR bacteria are difficult to treat. (What to Know About Antibiotic Sensitivity Testing, 2021)

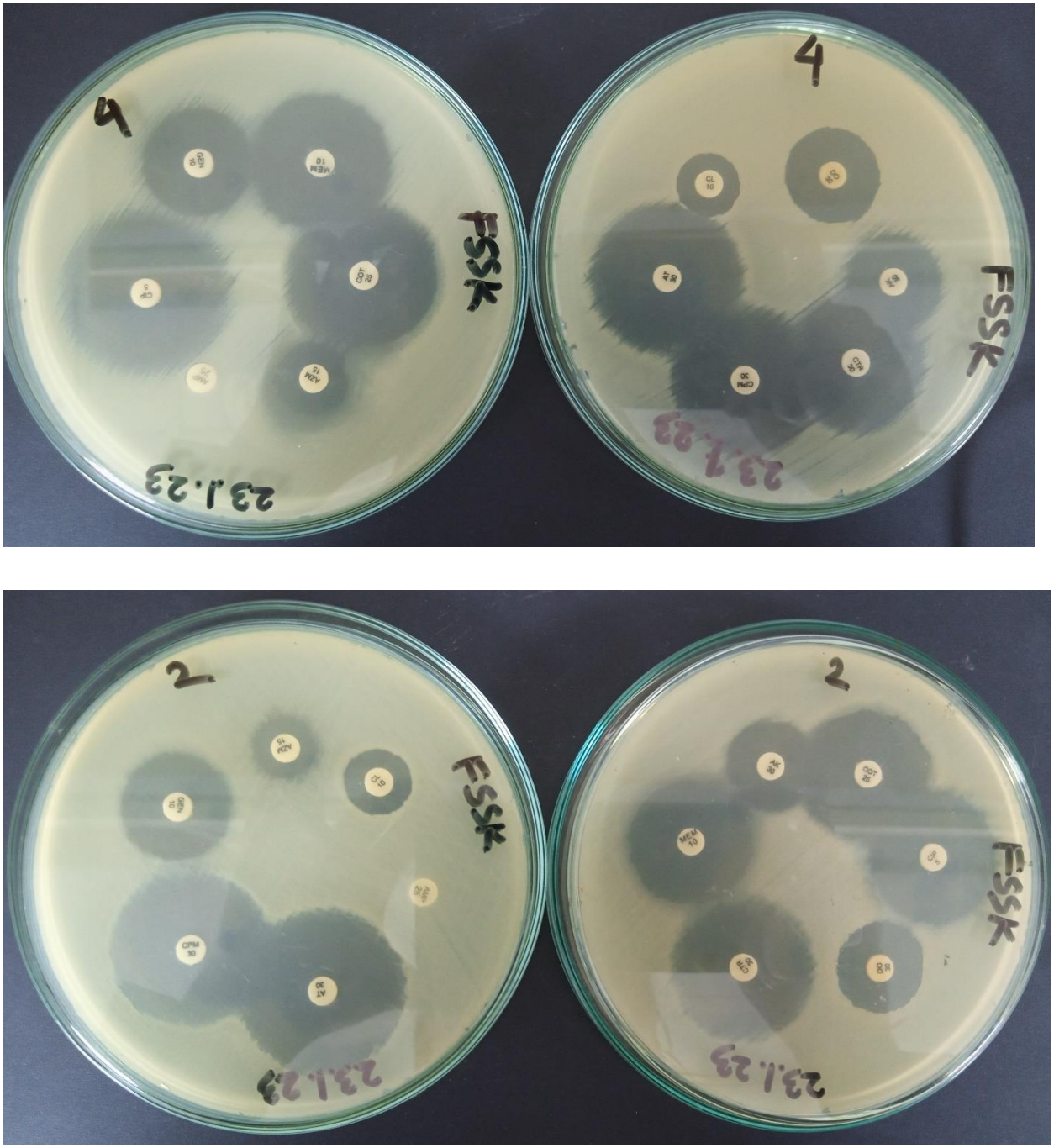


Figure 2.11: Antimicrobial Susceptibility Test

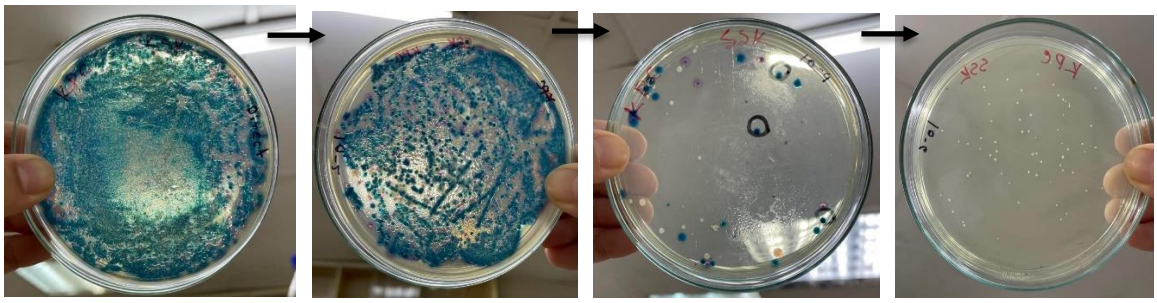
Chapter 3

Result & Observation

3.1 Summery of the Result and observation:

Selecting isolates for DNA extraction and PCR:

The duration of our study was June2022-November2022. In this period, we collected 57 water sample, among them 14 samples were collected from hospital effluents and 43 samples were collected from their adjacent community's tap water. Both hospital and community samples (after enrichment) were serial diluted to get different concentration which helped to obtain best discrete colony results on spread plate technique. Colonies were appeared in the selective media according how much dilution factor were spread. Usually 10^-2 to 10^-6 dilution factor were used to spread. Colonies were used to appear like 10^-2>10^-4>10^-6.

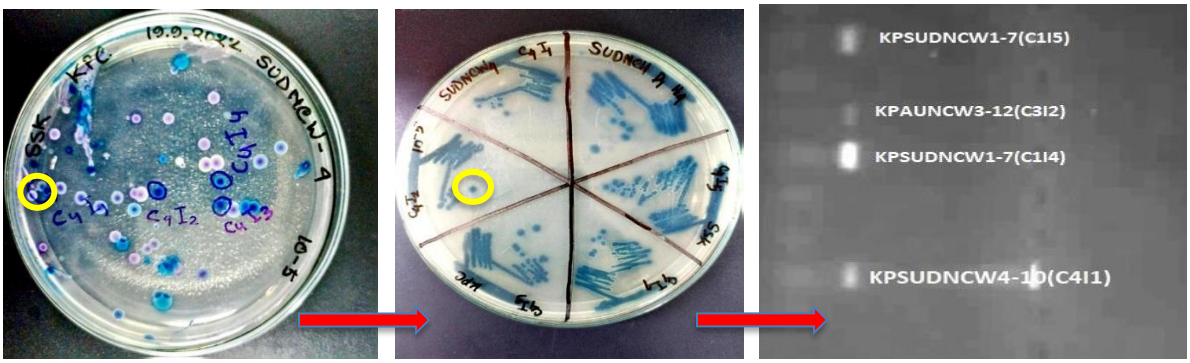


Dilution factor: **Direct** Dilution factor: **10^-2** Dilution factor: **10^-4** Dilution factor: **10^-6**

Figure 3.1: Spread plate results of JUDNH according to dilution factors on KPC media

After observing the spread plate results, we select discrete colonies from the plates to streak them into selected media. 2-4 colonies were used to selected from each plate.

Later, colonies that were obtained from streak results were go for DNA extraction. 40 colonies from Hospital water sample and 123 colonies from community water sample were taken for DNA extraction and later PCR. After PCR or polymerase chain reaction gel electrophoresis were done.



Selected isolated colony for streaking: - KPSUDNCW4-10(C4I1)

Single colony were obtained for DNA extraction and PCR

PCR positive KPSUDNCW4-10(C4I1) showed band on gel electrophoresis result

After electrophoresis, the amplified gel products were examined with a UV light source. The isolate was then compared with the specified band size to ascertain positive. The accompanying figures use UV lighting to display the PCR-amplified products.

Month wise summary of the PCR confirmed:

If we summarize total PCR confirm isolates throughout the months of June2022 to November2022, we can observe that 44 PCR confirmed were found in the time period. Among them 17 PCR confirmed were from hospitals and 32 confirmed were found from community water sample.

In particular, the time period from June 2022 to December 2022 was the focus of the study's observation of trends in Klebsiella pneumoniae. Based on its shape and criteria for amplified band size, Klebsiella pneumoniae was presumptively identified and validated using PCR. According to the findings, the maximum number of Klebsiella pneumoniae isolates—19 in all—were discovered in September 2022, accounting for 35.3% of all PCR-confirmed isolates. Only 5.9% of verified isolates were found in August 2022, the lowest number ever. Furthermore, 11.8% of Klebsiella pneumoniae isolates were discovered in October 2022, while 23.5% were detected in July and November 2022. The pie chart that comes with this article shows how these results were distributed.

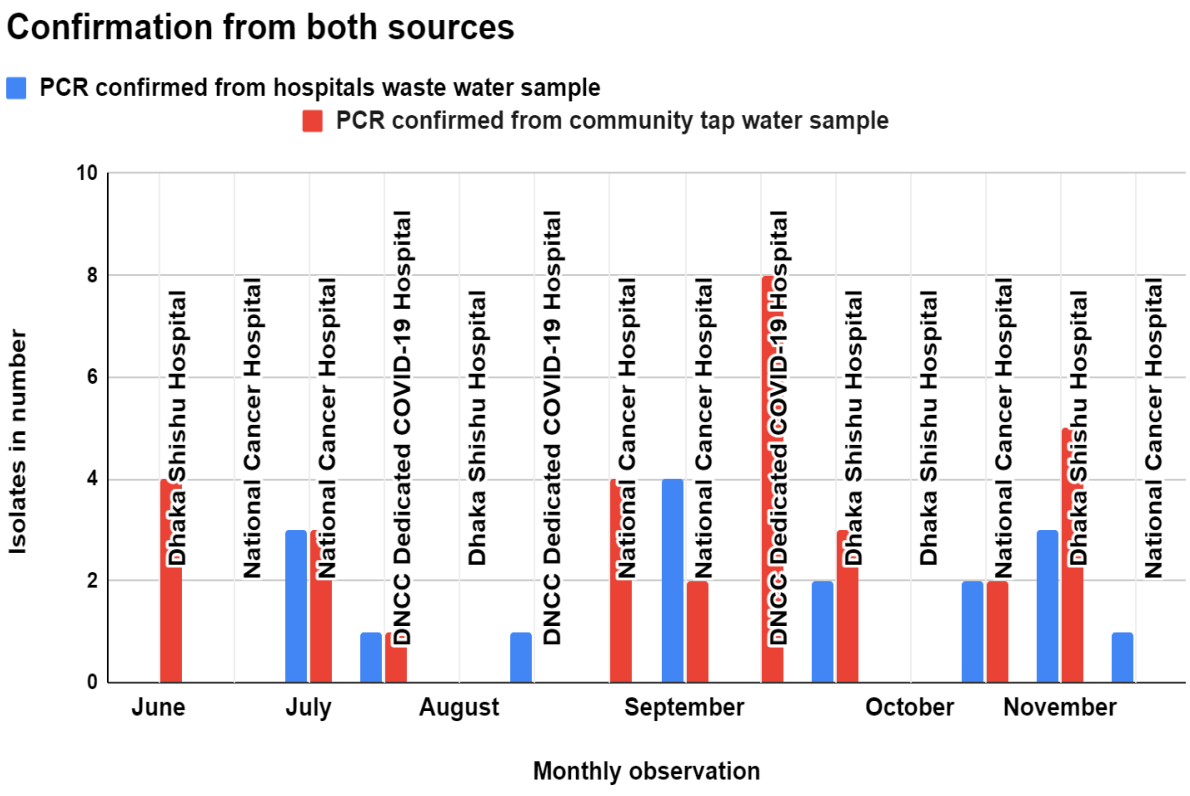


Diagram 6: Bar chart of total PCR confirmed isolates

Summary of the PCR confirmation:

The total confirmed PCR count was 49. After electrophoresis, the amplified gel products were examined with a UV light source. The isolate was then compared with the specified band size to ascertain positive. The accompanying figures use UV lighting to display the PCR-amplified products. They showed clear band on gel electrophoresis.

Figure 3.2.: PCR positive band shows in the gel electrophoresis under UV light

Summary of total Hospital water sample and community water sample target organism detection:

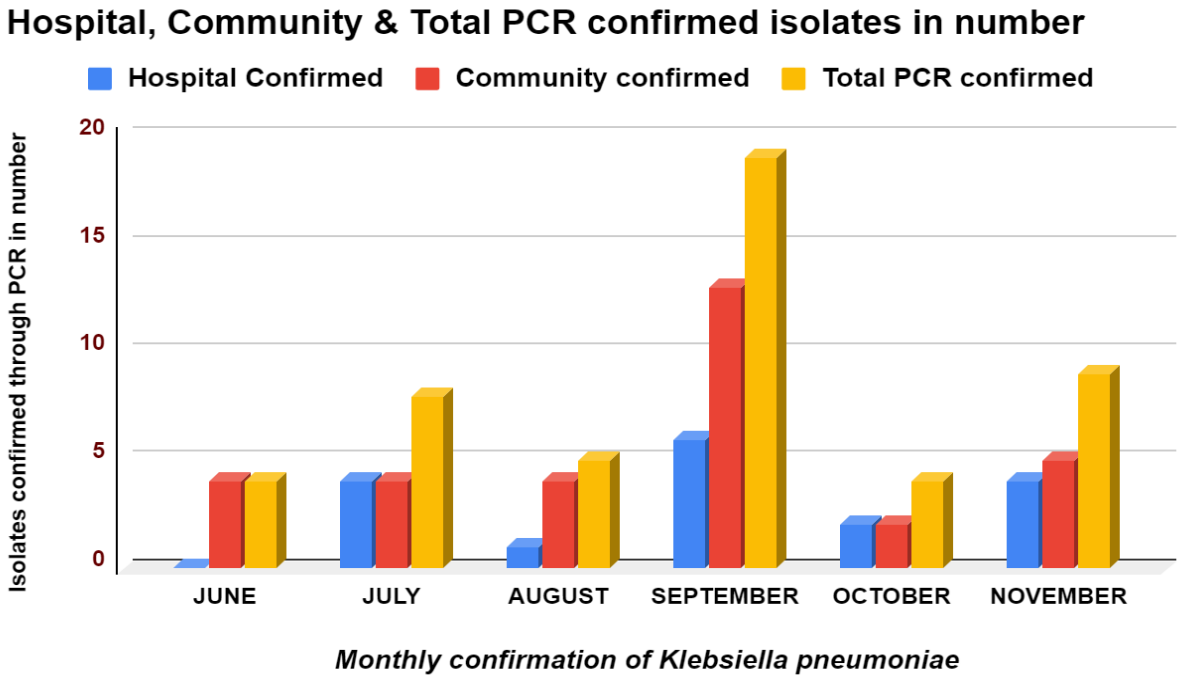


Diagram 7: Bar chart for monthly detection of confirmed isolates

This bar chart shows that, the lowest suspected organism was found in the month of August2022. The second lowest will be June2022. Highest number of suspected organisms found in September2022 from DNCC- DNCC Dedicated COVID-19 Hospital. After that, second highest hospital from where suspected organism were found was National Cancer Hospital. In the month of July2022, October2022 and November2022 the sample were collected from only two hospitals. The suspected and confirmed isolated were almost similar for all three months.

3.2 Antibiotic Susceptibility Testing

Resistance and sensitivity pattern of confirmed isolates:

Table 1: Table of zone interpretation according to CLSI standard guidelines-

Antibiotic Name	**Antibiotic Class**	**Zone Interpretation (mm)**
Gentamicin (10) (GEN)	Aminoglycosides	S>=15, I-13-14, R<=12
Amikacin (30) (AK)	Aminoglycosides	S>=17, I=15-16, R<=14

Cefixime (5) (CFM)	Cephalosporin	S>=19, I=16-18, R<=15
Ceftriaxone (30) (CTR)	Cephalosporin	S>=23, I=20-22, R<=19
Cefepime (30) (CPM)	Cephalosporin	S>=25, I=19-24, R<=18
Meropenem (10) (MEM)	Carbapenem	S>=23, I= 20-22, R<=19
Ciprofloxacin (5) (CIP)	Fluoroquinolones	S>=26, I=22-25, R<=21
Azithromycin/ Erythromycin (15) (E)	Macrolides	S>=23, I=14-22, R<=13
Doxycycline (30) (DO)	Tetracyclines	S>=>14, I= 11-13, R<=<10
Amoxiclav (30) (AMC)	Amoxicillin & Clavulanic Acid	S>=18, I= 14-17, R<=13
Chloramphenicol (30) C	Cephalosporin	S>=18, I= 13-17, R<=<12
Aztreonam (30) (AT)	Monobactam	S>=21, I= 18-20, R<=17

Source: CLSI guideline for disc diffusion AST 2020

In this research one of the main objectives was to monitor sensitivity and resistance of target organism *Klebsiella pneumoniae*. From the zone diameters of AST from our monthly isolates represent our findings where we have observed sensitivity towards most of the antibiotics used for almost 90% isolates. Now, that is in fact a good news that even though our effluent treatment and supply systems are not constructed with appropriate plans or in reality because of waste treatment cost, a lot of industries specially avoid this part and somehow just maintain a very basic effluent treatment system like settling treatment, COD/BOD ratio, Disinfection (Sultana et al., 2013) which are low in cost but lowest in impact to control most of the hazardous wastes. But for hospital waste water treatment in Bangladesh before December of 2005 the waste treatment process was a matter of ignorance for most of the health care facilities. The wastes produced were not even segregated for almost 80% of HCF. In contrast, generally hospital wastes were supposed to be segregated individually according to the categories of waste management of EIA (Environmental impact assessment). Also, what is even worse and unfortunate is this that some of the hospitals did separate their wastes based on whether the waste is infectious or not at the waste streamlet but all those streamlets ended up getting mixed up with all other wastes of DCC dustbins where the wastes are considered to be managed before letting most of them into the drainage. In general, all the wastes of pharmaceutical industries such as inhalers, spray cans or other pressurized containers were used to dispose all together with the regular produced wastes. As a result of mixing of both infectious and non-infectious wastes not only makes the pressure of environmental risks but also the ecosystem and its balance with human lifestyle, health, food and so on (Hassan et al., 2008). Therefore, all these evidences bring the concern and focus to the connection of any type of probable spread of pathogenic organisms from hospital wastes to nearby communities where

people get their regular use of water supply line and so this research conducted the antimicrobial susceptibility test for all the confirmed isolates. In the result, (showed above) it is observed that the isolates from both hospital and community were completely sensitive to the aminoglycosides such as, Gentamicin, Amikacin and carbapenem (Meropenem) and mostly sensitive to other antibiotics such as, cephalosporines, tetracyclines, fluoroquinolones and monobactam. But most resistances were found in macrolides group (Azithromycin/ Erythromycin) and then cefixime and Amoxiclav respectively. According to all these data a chart is attached below for better analysis.

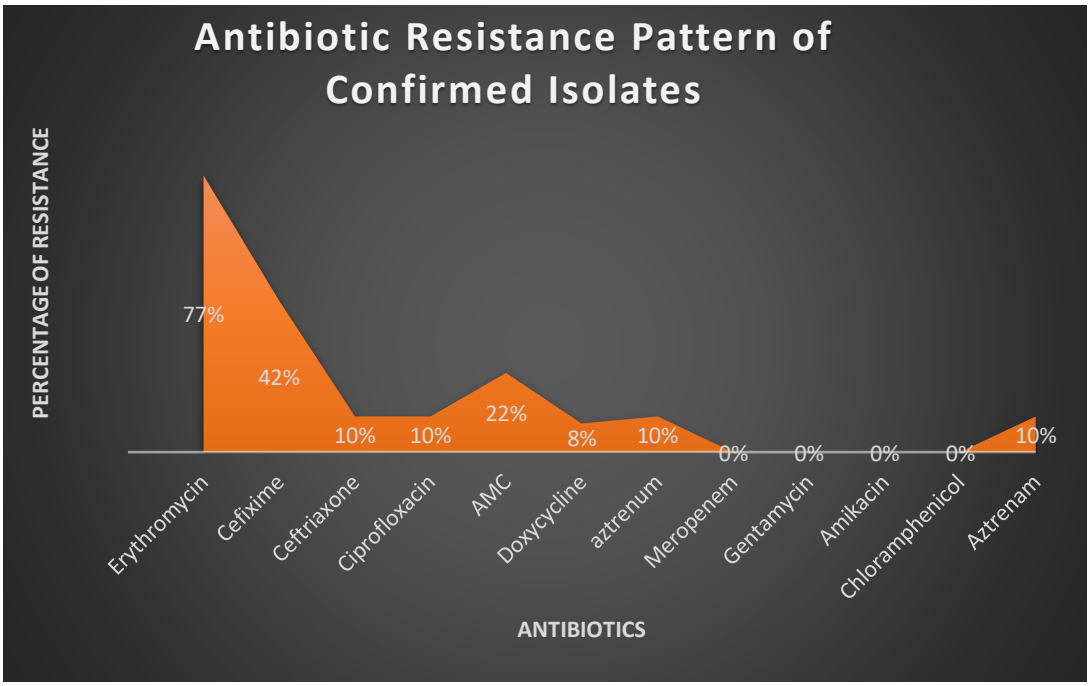


Diagram 8: Antibiotic resistance pattern of confirm Isolates

Anyway, to conclude this part, it is safe to say that even after treating our own environment so inappropriately still we are left with hope and opportunities to reprogram all the mistakes made so far and save our future before it's too late.

For the surveillance of our study purpose a table and a diagram based on that table information of comparison between confirmed hospital isolates AST result and confirmed community isolates AST result is given below-:

Table 2: AST result comparison between hospital & community

Antibiotics	Hospital Resistant	Community Resistant	Hospital Intermediate	Community Intermediate	Hospital Sensitive	Community Sensitive
Gentamicin (10)	5%	0%	0%	0%	65%	100%
Amikacin (30)	5%	0%	0%	0%	65%	100%
Meropenem (10)	0%	0%	0%	0%	100%	100%
Cefixime (5)	80%	37.5%	5.8%	3%	47%	59%

Ceftriaxone (30)	29%	0%	0%	3%	70%	100%
Cefepime (30)	23%	0%	0%	0%	70%	100%
Ciprofloxacin (5)	11%	9%	0%	9%	88%	84%
Azithromycin/ Erythromycin (15)	82%	75%	0%	0%	17%	25%
Amoxiclav (30)	23%	21%	17%	3%	58%	75%
Doxycycline (30)	6%	9%	6%	0%	88%	90%
Chloramphenicol (30)	0%	0%	0%	0%	100%	100%
Aztreonam (30)	23%	3%	0%	0%	76%	97%

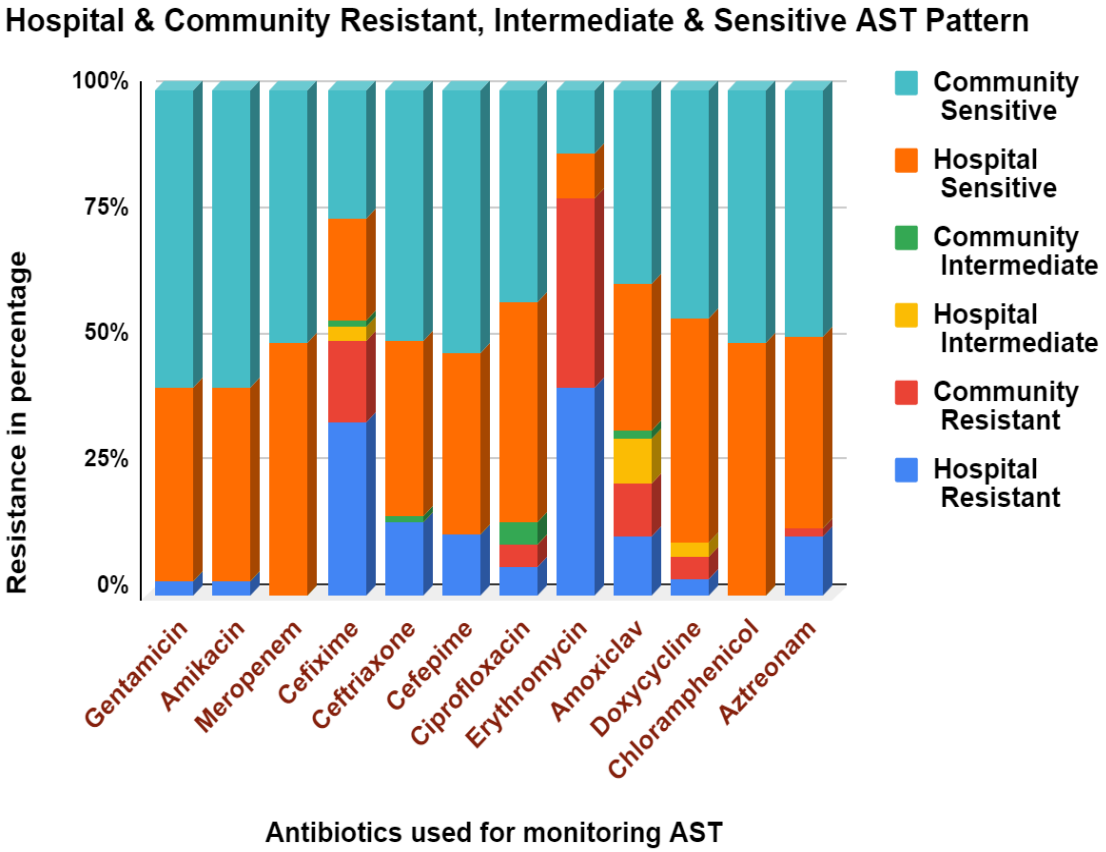


Diagram 9: Comparison between hospital & community AST pattern in percentage

A comparison between hospital & community isolates non-susceptibility (Both Intermediate and Resistant) AST pattern is shown below-

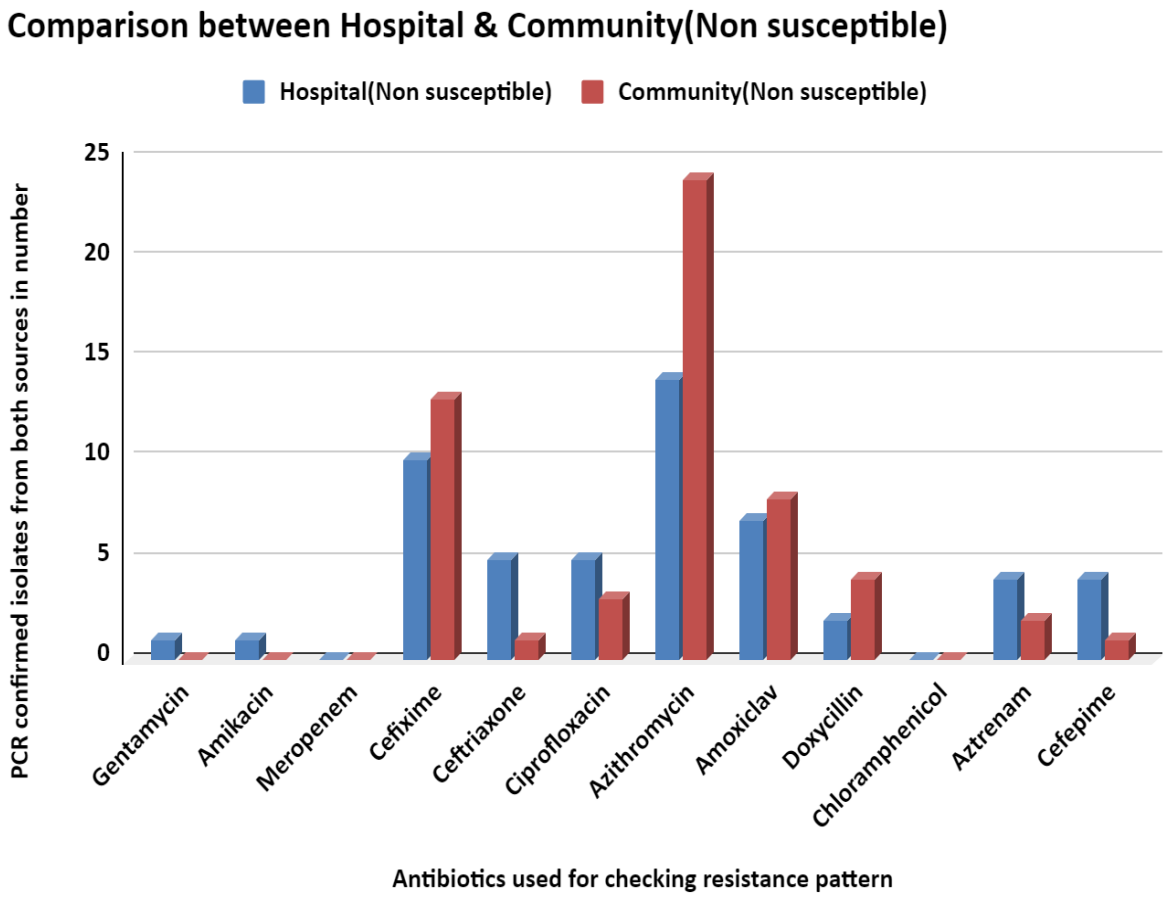


Diagram 10: Comparison between non-susceptible isolates from both sample sources in number

Chapter 4

Discussion and Conclusion

Discussion

Antibiotics can be classified as narrow and broad spectrum. Narrow-spectrum antibiotics are usually limited in their function as they are only effective against gram positive bacterial infection and to some extent in a shorter range some of these antibiotics can be useful against gram negative bacteria. The commonly used narrow-spectrum antibiotics are- bacitracin for treatment of Gram-positive infections such as and polymyxins for the treatment of Gram-negative infections.

On the contrast, Broad-spectrum antibiotics act against both Gram-positive and Gram-negative bacteria which also includes a different variety of organisms like- protozoa, fungus, parasites, virus etc. These antibiotics can be employed at emergency whether the exact infective causative bacteria have been identified or not. Therefore, these antibiotics might express some side effects although they have a greater impact on bacterial flora infected the body. Common examples of broad-spectrum antibiotics are- azithromycin, amoxicillin, tetracycline, quinolones.

However, if it's not so necessary, the broad-spectrum antibiotics should not use extensively. Because of their greater activity they can negatively affect the normal micro flora of the body which are helpful to human body. These normal micro florae compete with the invading microbes for nutrients and prevent them from growing rapidly. But overdose and frequent use of these types of antibiotics will destroy both the invading pathogen as well as normal micro flora which may provide advantage to the surviving real pathogen and might provide chances to some of the common organisms of normal micro flora to become opportunistic pathogen themselves! As a result, they might also contribute to the spread of antibiotic resistance genes among future invading pathogens. So, if it's not too important, the use of broad-spectrum antibiotics should keep restricted for the welfare of human being.

Klebsiella pneumoniae is a gram-negative bacterium which is pathogenic to human body. However, they are the causative agent of various types of human disease including pneumonia, bronchitis, and UTI. However, *Klebsiella pneumoniae* was the focus of our research, and so, we set out to locate and isolate it from both hospital waste water and public water supplies so that we could examine its Antibiotic Susceptibility pattern across a variety of antibiotic classes.
During the research period (June 2022- November 2022) 57 water samples were collected from 3 different locations of hospital and community. Among them, the numbers of positive isolates were 49. It was also found that, community water sample confirmed more positive isolates which were 32 in number than hospital waste water sample which were only 17. For the community water sample, we used buffer peptone water for the enrichment to increase the concentration of all the bacteria that are present in the sample. However, it is a non –selective broth which has a high pH. However, it might be a question that why lysogenic broth or nutrient broth was not used for the enrichment. LB broth is commonly used for *E. coli* and NB broth mainly focuses on Salmonella.

spp. However, it had been already discussed that BPW is a non-selective broth. This is why in our study; we chose BPW for pre-enrichment. By polymerase chain reaction, the isolates were confirmed. PCR is the most important application of modern science. Nowadays, laboratories, research centers are more reliable for their work on this method. A study was performed for in new Delhi on, the comparison to analyze the samples of wastewater from hospital regions and to bring the comparison in a scientific way, samples from residential areas or in other words community water was included to track the potential source that is responsible for the spread pathogenic organisms in Dhaka, Bangladesh. The majority of bacteria were *Klebsiella pneumoniae* (44%) (Islam et al., 2017)

All around the world, emerging resistance of different antibiotics in an excessive level gives a sign of invisible pandemic that might occur anytime when none of the available antibiotics will work and the morality rate will be the highest due to untreatable infections of MDR pathogens. However, the treatment capability of common infectious diseases is now in the frontline of threat to become unworthy of saving human lives by the raising resistance mechanisms of microorganisms globally. As a result of decreasing effectivity of antibiotics, a lot of cases are getting harder or more accurately to put the word is impossible to treat which involves rising number of infections by pathogens that were previously sensitive to even penicillin but now 3rd generation or even combined drug therapies are becoming challenging, including gonorrhea, pneumonia, blood poisoning, tuberculosis and so on. Increasing numbers of *Klebsiella pneumoniae* strains with resistance to multiple antibiotics have been found in sewage from hospitals and other potable water. (Giri S;Shekar M;Shetty AV;G PT;Shetty AK;). However, our study also focused on antibiotic susceptibility patterns of the positive isolated bacteria. Multidrug-resistant prevalence *Klebsiella pneumoniae* rates were over three times higher in 2016, 2017 and 2018 (20.6%, 20.5%, and 19.6%, respectively) than the European mean for the same time period. (Mijović et al., 2020).

From our antibiogram result, it was found that *Klebsiella pneumoniae* was mostly resistant to Azithromycin/Erythromycin which was 77%. However, the second resistant antibiotic was the 3rd generation cephalosporin which is cefixime (42%). A study in an article claimed that, *K. pneumoniae* resistance to third-generation cephalosporin in Montenegro is higher than that in certain neighboring countries, including Bosnia and Herzegovina (71%), Serbia (85%), and Northern Macedonia (95%), according to the most recent data from the WHO for 2018. (Mijović et al., 2020). In our study, analyzing the whole AST result including both community and hospital isolates together, two types of 3rd generation cephalosporin was used including cefixime and ceftriaxone. It should be noted that, the antibiotics showed different resistance numbers whereas cefixime showed 42% resistance level against *Klebsiella pneumoniae.* On the other hand, ceftriaxone showed 10% resistance level against *Klebsiella pneumoniae.* The 4th generation of cephalosporin cefepime showed 10% resistance against the isolated organism. The Fluoroquinolones showed 10% resistance. However, penicillin, Amoxiclav (Amoxicillin + clavulanic acid) showed 22% resistance. Then, doxycycline showed 8% resistance. Moreover,

monobactam including aztreonam showed 10% resistance. This resistance percentage has been already represented in the result and observation section.

From community and hospital sample, the antibiotic resistance pattern was different. From hospital sample gentamicin was 5% resistant and from community sample it was 0%. However, both from hospital and community sample it was 0% intermediate. Additionally, they showed different sensitivity pattern. The hospital isolates were 80% resistant against cefixime. On the other hand, hospital adjacent community samples showed 37.5% resistant against the antibiotic. Therefore, the result is still relieving according to the findings that we still have options for treatment specially for patients with critical conditions for example, cancer, diabetes, dialysis patients and other chronic disorders including immunocompromised patients with genetic disorders.

In our study, surprisingly it was found that *Klebsiella pneumoniae* was not resistant to carbapenems (meropenem) 0%, aminoglycosides (amikacin, gentamycin) 5% and chloramphenicol 0%. However, most of the studies done before regarding *Klebsiella pneumoniae* shows that this organism is constantly showing resistance to not only carbapenems but also beta lactam antibiotics. An important public health issue in Europe is the rising prevalence of strains *Klebsiella pneumoniae* resistant to carbapenems, with Greece having the highest rate (60.5%). (Sakkas et al., 2019). Even though we did not find any resistance against carbapenem drugs but we did find isolates showing highly resistance towards cephalosporins such as cefixime and others. So, it can be assumed that those organisms might be a source of spreading genes of these resistances among other strains as well as other organisms. As a matter of fact, it is indeed the high time to focus on our waste management, drainage system, pure supply system and an all-over improvement on the current situation. A relative table is shown below-

Table 3: Findings of this study & comparison with other relevant studies

Findings	**Relevant Studies**
Multidrug resistant pathogen *Klebsiella pneumoniae* were found in hospital wastewater and the adjacent community water.	A study claimed that, increasing numbers of *Klebsiella pneumoniae* strains with resistance to multiple antibiotics have been found in sewage from hospitals and other potable water.
The isolates were mainly resistant against erythromycin belongs to macrolide group and 3rd generation cephalosporin which is cefixime in terms of all the antibiotics used in the research.	A study in an article claimed that, *K. pneumoniae* is resistant to 3rd generation cephalosporin in Montenegro is higher than in certain neighboring countries, including Bosnia and Herzegovina (71%), Serbia (85%), and Northern-Macedonia 95%), according to the recent data from the WHO for 2018.

Conclusion

When it is about antibiotic resistance of *Klebsiella pneumoniae*, it is indeed one of the bacteria on upper list to worry about. Also, what's more, day by day it is increasing. In our study, we mainly focused on *Klebsiella pneumoniae* which is a significant antibiotic-resistant infection all across the world. The result of our research showed that among 57 samples from 3 different places of hospital and community we got 49 positive isolates of *Klebsiella pneumoniae*. However, antibiotic susceptibility test was done as well. From the antibiogram result, it was found that Azithromycin/ Erythromycin was mostly resistant to *Klebsiella pneumoniae.* Surprisingly, our research found that, *Klebsiella pneumoniae* was not resistant to carbapenems (meropenem), aminoglycosides (amikacin) and chloramphenicol even though most of the studies show that they are highly resistant to *Klebsiella pneumoniae.*

Bangladesh is a developing country and the situation of prescribing and using antibiotics it can be predicted that it will develop a high degree of antibiotic resistance in a short period of time, leading to global threat. In a study performed in Chittagong in 2003, typhoid patients were found to be unresponsive to second generation antibiotic therapy- ciprofloxacin. But first-generation antibiotic therapy had not even attempted because of their existing resistance. Therapeutic failures like these cases are very common in Bangladesh specially for multiple chronic disease patients! As a result, a significant number of patients are developing nosocomial infections, which is responsible for leading the condition to surgical failure, treatment failure and finally nothing but the end of life and close one's losing their beloved person even after trying in every possible way with everything they got! There are several reasons of this rapid surge in antibiotic resistance in this country. Some important reasons are mentioned below-

- Unnecessary and irrational antibiotic prescribing by physicians.
- Incorrect dosage intervals.
- Not completing the suggested dosage.
- Extensive misuse of antibiotics.
- Habit of self-medication among patients and normal people.
- Indiscriminate use of antibiotics in agriculture and farming in all over the country.
- Poor quality of drug production in local pharmaceutical industries.
- Inadequate surveillance of hospitals, health care institutes and pharmacies along with their waste management which is getting more attention nowadays because of MDR organisms.
- Lacking of the employment in terms of appropriate human force in necessary fields like- microbiologist, virologist, pharmacists in hospitals and clinics.
- Business priority (Industry, pharmacy) over public service- rising the price of drugs during an outbreak!
- Use of chemicals in agriculture, soil, foods and other manufactured products.
- Unplanned dumping of medical instruments, expired medicines.
- Improper hygiene practices.

- Overall deficiency of knowledge among mass people about medicines, healthy diet and maintenance.

According to a study it is found that antimicrobial resistance can be prevented in different ways which are shown in the diagram below (Uchil et al; 2014)

Diagram 11: Battle Against AMR

This diagram shows different types of strategies including maintaining hygiene, motivation to keep the environment clean, using the essential drug list etc.

Possible Prevention of AMR and waste management contamination:

- Infectious & non-infectious materials, particularly used and expired must be disposed according to high microbiological standard and tested prior to any operation.
- All equipment should be exposed & tested for preventive maintenance and should be properly cleaned before and after use to prevent cross-contamination between waste and supply systems.

- Since the environmental requirements vary according to the type of product being used as well as processed, they should be prepared according to the standard requirements.
- Staffs involved in the system development should have a clear knowledge of the importance of personal and production hygiene.
- Proper monitoring of critical control points and keeping the exact records and taking proper actions if any deviation has been noticed in the systems.
- Checking the validation and systemic identification of mechanical equipment and different sterilizing methods should be performed on a regular basis.
- Most importantly, mixing up of all the wastes or dumping all types of wastes in the same place for processing must be avoided to keep the undesired contamination away.
- Awareness among people is another important factor for preventing and controlling infectious diseases.
- In addition to that, immunization like vaccines must be provided and it is necessary to make people understand that these vaccines are for their own safety.
- Avoiding the antibiotics that are banned from international drug monitoring committees.
- Putting a full stop as much as possible in terms of self-medication and misuse of antibiotics and their dosage.
- Increasing the collaboration between international and local drug monitoring committees and policies.

Klebsiella pneumoniae is the causative agent of different types of dangerous disease. To prevent the certain types of disease it is necessary to keep clean. The hospital effluent must be kept clean so that it does not spread in environmental water. As antibiotic resistance is presenting significant threat to human health, it is now a necessary thing to monitor its environmental prevalence and spread. However, more effective chemicals and improved diagnostic technology are required. Also, the use of antibiotics properly is important which involves using them when there is bacterial infection. Then, completing the antibiotic course and never using the left-over antibiotics which initiates infection. Patients should only consume antibiotics when it is prescribed by doctor. So, only awareness and monitoring by authorization bodies are not enough to control any kind of infection, it needs awareness and self-hygiene practices and maintenance to complete the task. Therefore, we conclude our study here by presenting all our works, findings, observations and help from other related articles to justify, compare and complete the report.

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