COMPARATIVE ANALYSIS OF *ACINETOBACTER BAUMANNII* ISOLATED FROM HOSPITAL WASTEWATER AND ADJACENT COMMUNITY TAP WATER: INVESTIGATING ANTIBIOTIC RESISTANCE AND AMR GENES

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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

August 2023

Declaration

It is hereby declared that

1. The thesis submitted titled "**Comparative Analysis of *Acinetobacter baumannii* Isolated from Hospital Wastewater & Community Tap Water: Investigating Antibiotic Resistance and AMR Genes**" 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.

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Ethics Statement

For the completion of this study, samples from selected venues were collected following all necessary safety precautions. All the experiments needed for this project were performed at BRAC University Life Sciences Laboratory. It should also be noted that no animal or human models were used in this study.

Abstract:

Antibiotic resistant *Acinetobacter baumannii* (*A. baumannii*) has emerged as important nosocomial pathogen associated with various infections in clinical settings. It has the ability to persist in the environment for prolonged periods of time due to the wide range of antibiotic resistance factors it possesses. *A. baumannii* represents a global threat owing to its ability to resist most of the currently available antimicrobial agents. Hospital wastewater is considered to be one of the main reservoirs for antibiotic resistance because they create a system where antibiotic resistance (AMR) genes can be shared. Our study was performed to assess and analyse *A. baumannii* isolated from the wastewater of 3 selected hospitals and tapwater of houses surrounding the hospitals in order to create a profile of the antibiogram and antibiotic resistance genes of A. baumannii found from these environmental niches. 37 samples were collected from the sampling sites from March 2023 to June 2023 analysed presumptively by culture-dependent methods. A total of 100 isolates were confirmed to be *A. baumannii* by detection of the blaOXA-51-like gene. 52 representative isolates were undergone Antibiotic Susceptibility Testing and the highest antibiotic resistance profile was seen against Cefixime (100%), Ceftazidime (96.1%) and Aztreonam (84.6%). 100% of the isolates were seen to be multi-drug resistant (resistant to at least 3 antibiotics). Molecular screening of 6 beta-lactamase encoding genes was performed. Among the investigated genes, only the prevalence of blaNDM-1 was found in 5 isolates- 4 from hospital wastewater source and 1 from community tap water source. blaCTX-M, blaSHV, blaTEM, blaOXA-48, blaIMP were not detected in any of the studied isolates. 37.6% of imipenem resistant isolates showed the presence of blaNDM-1. The findings of our study revealed the availability of NDM-1 producing *Acinetobacter baumannii* strains in the environment setting. It can be expected that these antibiotic resistant genes would spread from hospitals via the untreated sewage effluents and disseminate to our supply water which is a threat for public health.

Dedication

I, Nafisa Mehreen Naser, would like to dedicate my Thesis to my most beloved grandmother, mother, younger brother and my teacher & supervisor Md. Hasannuzzan Sir (Senior Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University) without whose continual support, guidance, advice, and encouragement and prayers, I would not be able to complete such an essential part of my Undergraduate Degree.

I, Md. Safwan Sakib, would like to dedicate this thesis to my beloved parents and my family; without them, I am nothing. My friend and thesis partner, Nafisa Mehreen Naser, without her dedication and immense support it would not have been possible to complete this thesis. My university faculties, especially Md. Hasanuzzaman (Senior Lecturer, Microbiology Program, Department of Mathematics and Natural Science, BRAC University) and Akash Ahmed (Lecturer, Microbiology Program, Department of Mathematics and Natural Science, BRAC University), motivated and enlightened me with knowledge whenever I needed. I am forever grateful to each of them.

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Our Laboratory Officers- Md. Mahmudul Hasan, Shamim Akhter Chowdhury, and Asma Binte Afzal have always graced us with help whenever we needed for which we are grateful. And of course, the incomparable generosity and guidance of our Research Assistant Nishat Tasnim Ananna. Throughout the project, she has been by our side- assisting us, teaching us, figuring out how to set up our experiments, how to interpret our data and always being available for us no matter day or time. Having her as our RA filled us with confidence that we will be able to finish our research on a good note. We thank you for facilitating us with knowledge and wisdom that we will remember for a long time.

Lastly, but not the least we would like to express our gratefulness to lab attendants Ashik-E Khuda, Tanzila Alam, and office attendant Nadira Yeasmin for their unwavering help during these 6 months at the laboratory and everyone who assisted us and engaged with us in the laboratory.

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LIST OF ABBREVIATIONS

ADCs Acinetobacter-derived Cephalosporinases

AG Aminoglycosides

AGE Agarose Gel Electrophoresis

AMEs Aminoglycoside-modifying enzymes

AMR Antimicrobial Resistant

ARGs Antibiotic Resistance Genes

AST Antimicrobial Susceptibility Test

bp Base pair

BPW Buffered Peptone Water

CAP Community-acquired pneumonia

CFU Colony Forming Unit

CLSI Clinical Laboratory Standards Institute

COVID-19 CoronaVirus Disease 2019

CRAB Carbapenem-resistant *Acinetobacter Baumannii*

CSF Cerebrospinal Fluid

DNA Deoxyribonucleic Acid

DNCC Dhaka North City Corporation

EDTA EthylenediamineTetraacetic Acid

ESBLs Extended-spectrum Beta-lactamases

HGT Horizontal Gene Transfer

ICU Intensive Care Unit

IDSA Infectious Diseases Society of America

LAM Leeds Acinetobacter Medium

LPS Lipopolysaccharides

MBLs Metallo-β-lactamases

MCT Microcentrifuge Tubes

MDR Multidrug-Resistant

MHA Mueller–Hinton agar

MIC Minimum Inhibitory Concentration

NA Nutrient Agar

NICRH National Institute of Cancer Research & Hospital

OMP Outer Membrane Proteins

OMVs Outer Membrane Vesicles

OXA Oxacillinase

PBP Penicillin Binding Protein

PCR Polymerase Chain Reaction

PFGE Pulse Field Gel Electrophoresis

rpm Revolutions per minute

RPPs Ribosomal Protection Proteins

T1N1 Tryptone Salt Agar

T2SS Type II secretion system

T6SS Type VI secretion system

TE Tris-EDTA

TBE Tris-borate-EDTA

UV Ultraviolet

VAP Ventilator-Associated Pneumonia

WHO World Health Organization

CHAPTER 1: INTRODUCTION

Antimicrobial resistance and its worldwide spread is one of the main issues and major challenges that we are facing in the 21st Century. Infections that are caused due to pathogenic strains that develop resistance to widely used drugs are expected to cause approximately 300 million premature deaths by the year 2050. (Vrancianu et al., 2020) Many strains of bacteria can be resistant innately or develop resistance through acquiring resistance genes in various ways. One of the main causes of the rapid increase of microbes becoming resistant is the blatant and escalating use of the revolutionary antimicrobial drugs, i.e antibiotics. (Monserrat-Martinez et al., 2019) This exploitative and abusive use of antibiotics in households and in hospitals without proper authorization therefore results in adverse clinical implication such as pathogenic bacteria becoming resistant to the common antibiotics that was able to treat these infections before- due to mutation. (Mapipa et al., 2022b)

Extended interaction with sub-lethal doses of antibiotics generates an environment for the natural selection of bacterial strains resistant to commonly used antibiotics which spreads, resulting in an increase in hospital acquired infection. (Mapipa et al., 2022b) Moreover, considering the fact that antibiotic resistance genes are highly mobile and can disseminate from one space to another (human to animals/sewage to environment), it is quite difficult to manage the pathway of spread (Vrancianu et al., 2020) rather looking into the core problem would be beneficial in the long run.

Bacterial resistance to antibiotics is common in areas where antibiotics are commonly used, i.e the hospital environment. This makes hospital wastewater an important source and hotspot of antibiotic-resistant bacteria, antibiotic resistance genes, and other medicines that enter the natural environment by dissemination from wastewater. (Hubeny et al., 2022c) Resistance determinants can be transferred into DNA of bacteria via Horizontal Gene Transfer which can further disseminate into natural bacterial communities and evolve them or make them pathogenic. (Ferreira et al., 2011) It is significant that in the last 20 years, there has especially been a global rise of bacterial infections caused by bacterial strains resistant to beta-lactams- mostly to 3rd generation of cephalosporins and carbapenems. (Safari et al., 2015) The most notable example of this would be Gram-negative opportunistic pathogens. In our research, we focus on one of these pathogens, namely *Acinetobacter baumannii*.

Acinetobacter baumannii is a Gram-negative pathogen, with traits such as being a lactose and glucose non-fermenter, non-motile and aerobic- and is one of the most frequently detected multidrug-resistant bacteria. (Vrancianu et al., 2020) The bacteria belongs to the genus

Acinetobacter that are frequently located in both soil and water. The majority of human *Acinetobacter* infections are caused by *A. baumannii*. It is reputable in its association with the infections it caused during the Iraq-Afghanistan war to US millitary soldiers which earned it the name "Iraqibacter". (Vrancianu et al., 2020) *A. baumannii* can withstand and survive harsh environmental conditions making them adaptable and easy to spread in various environmental niches. (Ferreira et al., 2011) Due to its high-spec virulence and resistance factors, ability to form biofilm and ability to colonize in a wide range of temperature and pH, it is able to cause various infections in the hospital setting in immunosuppressed patients, particularly those with a history of prolonged hospitalization such as pneumonia, meningitis, urinary tract infection, blood infection, soft tissue and skin infections, etc making it one of the top nosocomial pathogens, especially in the ICU. (Mapipa et al., 2022b)

Due to its rapidly developing resistance to first-line antibiotics, it is one of the most trouble-causing pathogens that is jeopardising the current antibiotic era. (Peleg et al., 2008) Carbapenems have been the primary drug of choice to treat multidrug resistant *A. baumannii* infections in many cases but with the emergence of carbapenem-resistant *A. baumannii*, options to treat its infections are greatly reduced.

So far, studies on antibiotic resistance patterns and identification of multi drug resistant *A. baumannii* have mainly been focused on clinical samples or wastewater. However there is a lack of available data on linking the transmission of such strains from hospital wastewater to our community supply water via dissemination. In such a case, to get a comprehensive surveillance of antibiotic resistance patterns, it is important to not only survey clinical isolates but compare wastewater isolates with environmental isolates around humans for a proper antibiotic stewardship program to be enforced. Thus, our study pulls inspiration from these questions and seeks to bridge the gap of knowledge about the prevalence of *A. baumannii* in hospital wastewater and community supply waters of a certain region of Dhaka. In order to achieve this, the prevalence of *A. baumannii* in the wastewater of selected hospitals, antibiotic resistance profile against selected antibiotics was assessed and the isolates were screened for the presence of several antibiotic resistant genes.

CHAPTER 2: LITERATURE REVIEW

2.1 *Acinetobacter baumannii*

Acinetobacter baumannii is a Gram-negative, aerobic bacteria which is also non-motile. It cannot ferment glucose or nitrate and shows negative in oxidase and indole tests. In terms of shape and morphology, it is a bacillus and takes the shape of short, broad rods in the rapid growth phase but assumes a more coccobacillary shape in the stationary phase of its growth. *A. baumannii* are one of the leading causes of fatal hospital-acquired infections (Mapipa et al., 2022), i.e hospitals acquired infections. These infections include ventilator-associated pneumonia, infections at regions that underwent surgery, urinary tract infections, and septicemia that are caused by *A. baumannii* globally. *A. baumannii* can be said to be an aquatic organism that thrives in water-based environments. It is also a natural inhabitant of soil. (Fidsa, n.d.) Overall, *A. baumannii* is ubiquitous in nature and can be isolated from soil, water, animals, and humans alike. In recent times, *A. baumannii* is one of the most abundantly encountered multidrug-resistant (MDR) bacteria often resistant to last-line antibiotics such as carbapenems. It once used to be a low-risk pathogen but now it has now risen as the main source of infections from hospital and community source. It is also an opportunistic pathogen that is one of the pathogens of the ESKAPE group categorized by the Infectious Diseases Society of America (IDSA). Since 2017, The World Health Organization (WHO) has declared *A. baumannii* as a critical pathogen of top priority. (Muzahid et al., 2023)

A. baumannii was first isolated by Dutch microbiologist Beijerinck in 1911 from soil with the use of a calcium acetate-enriched minimal media. (Howard et al., 2012) Now, due to how frequently it causes hospital outbreaks or infrequent acute infections in humans, the bacterium receives a lot of attention in the research sector. (Hubeny et al., 2022b) It can survive harsh environmental conditions, such as desiccation and higher then room temperature and extreme pH, so managing its infection in ICUs and burn units is a difficult feat. (Hamidian & Nigro, 2019) It also has a high biofilm forming cabability increasing its morbidity and mortality and well as serves as an added virulence factor. Various researchers have found *A. baumannii* to be able to form biofilms on surfaces of materials- biotic and abiotic alike. (Eze et al., 2022)

2.2 Diseases Caused by *A. baumannii*

On a global scale it was found that *Acinetobacter baumannii* is highly responsible for life threating nosocomial infections. It is known as a multidrug resistant organism due to its unbelievable capacity of acquiring resistant mechanisms. *Acinetobacter baumannii* can spread as a nosocomial

pathogen to the critical ill patients. This results in uprising morbidity and mortality. (Moubareck & Halat, 2020) In recent years, infections with *A. baumannii* has become a dangerous issue in hospitals because of it is causing diseases in urinary tract, skin and soft tissue even on central nervous system. (Moubareck & Halat, 2020)

Some major diseases it causes are:

Respiratory Illnesses: *A. baumannii* causes ventilator-associated pneumonia (VAP), and it is seen that it has a higher death rate in critically ill patients. (Moubareck & Halat, 2020) According to a research, *A. baumannii* is responsible for 8% to 14% of VAP and this percentage grows high to 19% to more than 50% in Asia, Latin America, and several Middle Eastern countries. (Moubareck & Halat, 2020) *A. baumannii* is also responsible for community-acquired pneumonia (CAP) and it has more fatality rate. It mostly affects people who has habits like alcohol consuming, smoking. Also to patients who has diabetes and chronic lung diseases. (Moubareck & Halat, 2020)

Bloodstream Infections/ Septicemia: *A. baumannii* is now one of the most common causes of bloodstream infections in the hospitals with the respiratory system apparatus or intravenous catheters. Bloodstream infections due to *A. baumannii* now becoming lethal day by day. (Moubareck & Halat, 2020) *A. baumannii* is showing high prevalence of bacteremia in cancer patients. According to a Brazilian research, this pathogen was responsible for almost 68% case of bacteremia. (Moubareck & Halat, 2020) From another study, is was found that *A. baumannii* is the most common pathogen in bloodstream infections in patients who were badly burned and admitted into ICU. (Moubareck & Halat, 2020) According to a research of Wareham, an investigation among 399 bacteremia infection samples were took place and *A. baumannii* was the most frequent isolated species. Most of them were from ICU and were multidrug resistant. Also with the resistance of carbapenem from 0% to 55%. (Wareham et al., 2008)

Skin and Soft Tissue Infections: *A. baumannii* can cause wound infection in wounded and burned patients. Which results in complications like slow healing of wound, failure in skin grafting and in more severe case sepsis and amputation. (Zurawski et al., 2019) There are many cases in war where injured soldiers were reported with *A. baumannii* in their wound. For example, in the military operation held in Iraq in 2003-2005, it was documented that *A. baumannii* has caused osteomyelitis and battle wound infection. Another study shows that, *A. baumannii* was the most often isolated bacteria from open tibial fractures in US military injured soldiers during war of Afghanistan or

Iraq. (Moubareck & Halat, 2020) Over the course of eight years, *A. baumannii* isolates increased its percentages from 4% to 55% in a US military hospital. (Moubareck & Halat, 2020)

Urinary Tract Infections: From a recent research it was found that one in five *A. baumannii* strains are isolated from urine location. (Moubareck & Halat, 2020) Generally, *A. baumannii* can cause infections in the urinary tract with the association of urinary catheters or percutaneous nephrostomy. (Bagińska et al., 2021)

Meningitis: Most of the time *Acinetobacter baumannii* meningitis can develop after neurosurgery. Because generally it is linked with the exposure of foreign apparatus like external ventricular drain. It has also a fatal rate in morality. (Xiao et al., 2018) According to a research, this morality rate of *Acinetobacter baumannii* meningitis are nearly 70% in ICU. Especially patients with indwelling ventriculostomy tubes or CSF fistulae who are also receiving post-surgical antibiotic therapy. (Moubareck & Halat, 2020)

2.3 Epidemiology of *A. baumannii*

Acinetobacter baumannii has a significant dominance in the global epidemiology in recent years. It can be observed that *Acinetobacter baumannii* infections occurs mainly in patients of ICUs and other hospital settings, patients who experience trauma due to natural disasters and war outbreaks. There are many researches that show the incidence of *Acinetobacter baumannii* infections in the patients of ICU are increasing day by day worldwide. (Falagas & Karveli, 2007) Mainly in Asian and European hospitals *Acinetobacter baumannii* has higher dominance in spreading nosocomial infections also they have more extensive antimicrobial resistance profile. (Falagas & Karveli, 2007) Behind this antimicrobial resistance, using broad spectrum antibiotic therapy like carbapenems or third generation cephalosporins contributes to put selective pressure and evolution of resistance. (Maragakis & Perl, 2008)

A multidrug resistant *Acinetobacter baumannii* infection outbreak and environmental contamination was observed on materials like laryngoscope blades, catheters, patient lifting equipment and curtains. (Maragakis & Perl, 2008) These materials highlight the spreading of *Acinetobacter baumannii* from the medical equipment. There are also reports of community acquired *Acinetobacter baumannii* infections. Mainly occurs in patients with co-morbidity like diabetes mellitus, chronic pulmonary diseases or alcohol addiction. They are mainly from tropical or sub-tropical regions. So it suggests that humid environment has relation with *Acinetobacter baumannii* infections. (Falagas & Karveli, 2007)

Some clinical data shows that from the previously occurred *Acinetobacter baumanni* infection cases from the earthquake in Turkey, Marma in 1999 and war of Iraq, Kuwait and Afghanistan, these areas and these causalities have great impact on the outbreak of *Acinetobacter baumanni* infections. (Falagas & Karveli, 2007) During the Iraq war, *Acinetobacter baumanni* had led to multidrug resistant strains outbreaks in the US military hospitals located on Iraq, Kuwait and Afghanistan. In this Middle East region *Acinetobacter baumanni* has had its most impact and for this reason *Acinetobacter baumanni* was also known as "Iraqibacter". (Maragakis & Perl, 2008) During this Iraq war, military personnel who suffered from traumatic injuries have been recorded to develop osteomyelitis, bacteremia, respiratory infections and multidrug resistant *Acinetobacter baumanni* wound. (Maragakis & Perl, 2008) Later it was observed that the transfer of this affected community had spread these *Acinetobacter baumanni* strains from the military troops to the general population through health care system. (Maragakis & Perl, 2008)

There are multiple reports throughout the world that suggests antimicrobial resistant of *Acinetobacter baumanni* has increased in the clinical isolates. The antimicrobial resistance is more dominant in the clinical isolates from ICU patients in Asia, Europe and South American medical care systems. (Falagas & Karveli, 2007) On the other hand, less extensive antimicrobial resistance *Acinetobacter baumanii* isolates were reported from the ICU patients in the USA and Netherland. (Falagas & Karveli, 2007) For the detection of outbreaks of infections of multidrug resistant *Acinetobacter baumanii* and track inter-institutional, regional and global transmission, molecular genotyping based method like PFGE (Pulse Field Gel Electrophoresis) can be used. As a result genetic likeness of *Acinetobacter baumanii* can be analyzed. In acute care hospitals in places like New York, Argentina and the UK, researchers showed inter-institutional spread of carbapenem resistant *Acinetobacter baumanii* infections. (Maragakis & Perl, 2008) In another research through PFGE is was reported how pandemic of *Acinetobacter baumanii* strains were migrated from Brazil to Argentina. (Maragakis & Perl, 2008)

2.4 Bacterial Pathogenesis and Virulence of *Acinetobacter baumannii*

Acinetobacter baumannii causes numbers of infections including skin and soft tissue, bacteremia, pneumonia, urinary tract infections, meningitis etc. (Morris et al., 2019) Most of the cases it produces nosocomial diseases at the same time it can also cause community diseases. Behind causing infectious diseases there are numbers of virulence factors. (Morris et al., 2019)

Some of the major factors are:

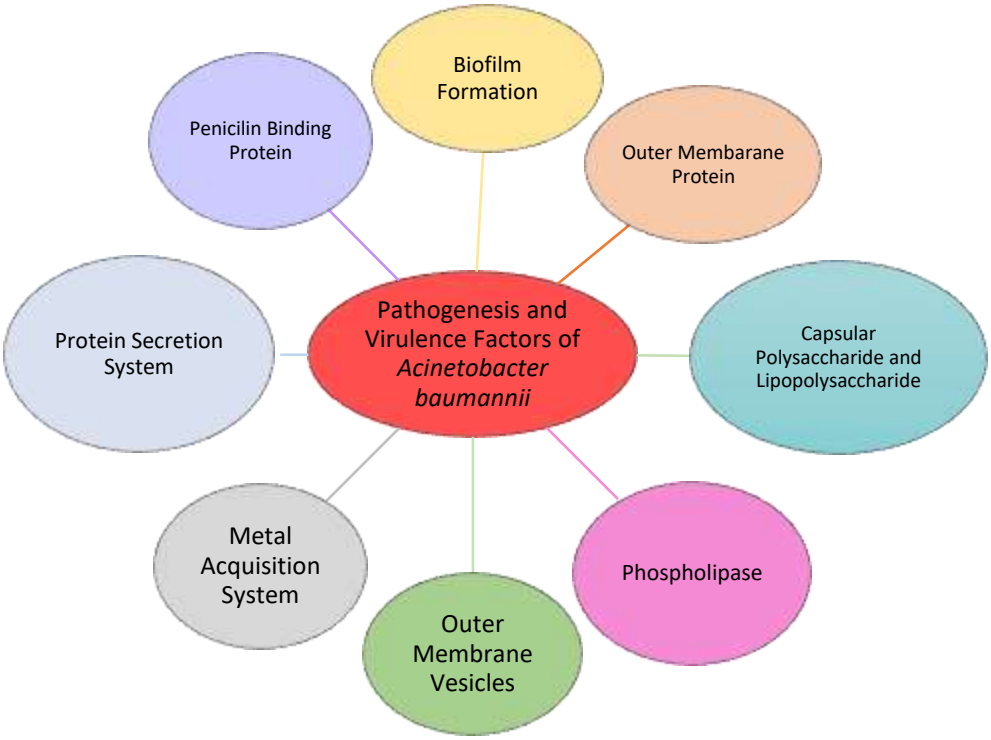


Figure 1: Pathogenesis and virulence factors of *Acinetobacter baumannii*

Biofilm Formation: Biofilms are mainly communities of microbes wrapped in an extracellular matrix. According to a research it was found that, the production of biofilms have interactions between *A. baumannii* and its host. It is also a key factor for infections that are linked to healthcare apparatus. (Harding et al., 2017) In majority of the cases *A. baumannii* develops biofilms on abiotic surfaces likewise medical equipment such as vascular catheters, endotracheal tubes, stainless steels and polycarbonate. (Harding et al., 2017) *A. baumannii* of biofilms are more durable to extracellular pressures. The adhesions, surface attachments, protective surface features like capsular polysaccharides are important elements for creation and maintenance of *A. baumannii* biofilms. (Harding et al., 2017) In comparison to low biofilm producing strains, high biofilm producing strains are less vulnerable to desiccation. (UpToDate, n.d.) Moreover, creation of biofilms appear to be a crucial factor for *A. baumannii* to survive under dry conditions. (UpToDate, n.d.) Additionally, the development of *A. baumannii* biofilms on non-living surfaces has a positive association with the expression of virulence factors and multidrug resistance thought to increase resistance 1000 times higher than in planktonic organisms. (Gedefie et al., 2021)

Outer Membrane Proteins (OMP): OmpA or outer membrane protein A is one of the most prolific virulence factor of *A. baumannii*. (Morris et al., 2019) Generally, OmpA is found in outer membrane vesicles or OMVs on time of usual growth and in vivo infections. From 103 clinical isolates 83 of them has shown more than 99% of sequence identity of OmpA while the most diverse had 85% sequence identity. Which suggests OmpA is highly conserved. (Morris et al., 2019) When OmpA interacts with eukaryotic cells by binding and adhesion to death receptors of eukaryotic cell surface, cytotoxicity is induced by eukaryotic cells. (Morris et al., 2019) According to a study, Omp34 which is one of the OMPs, is contributing the pathogenesis in *A. baumannii*. (Morris et al., 2019) In *A. baumannii* this Omp34 is also highly conserved while its presence is in more than 1600 strains with the identity percentage is greater than or equal to 98%. The interaction of Omp34 with the eukaryotic cells induces apoptosis. This apoptosis of eukaryotic cells is done by the restriction of autophagy and caspase dependent mechanism which promotes persistence of bacteria in autophagosome. (Morris et al., 2019)

Capsular Polysaccharides and Lipopolysaccharides (LPS): Lipopolysaccharide and capsular exopolysaccharide are another pathogenicity factors for *A. baumannii*. (C. R. Lee et al., 2017) There is a conserved gene cluster region in *A. baumannii* known as K locus. It is suggested that this K locus may regulate the capsular polysaccharide production. During the infection of *A. baumannii* interestingly many isolates from patients, expresses the surface capsular polysaccharide. (C. R. Lee et al., 2017) For capsule polymerization and assembly, ptk and epsA are two genes that are required from a prediction. (C. R. Lee et al., 2017) This ptk and epsA genes are also essential for growth in inflammatory of exudative fluid. In addition, pglC or pglL mutations cause atypical biofilm formations and attenuate lethality in a mouse septicemia model, which are caused by the synthesis of the O-pentasaccharide present on glycoproteins and capsular polysaccharides. (C. R. Lee et al., 2017)

Phospholipase: Phospholipases are recognized virulence factors. Two phospholipase C and three phospholipase D enzymes are encoded by *A. baumannii*. (Morris et al., 2019) Both of them are towards the phosphatidylcholine which is a membrane component of eukaryotic cells. All of the substrates of enzyme C and D are specific to phosphatidylcholine. (Morris et al., 2019) Both C and D enzyme work against erythrocytes of human by iron acquisition. Transcription of C and D enzyme is controlled by the ferric regulator also known as Fur. (Morris et al., 2019) For in vivo pathogenesis, resistance of serum and invasion of epithelial cell three phospholipase D genes are required. In addition, a gene duplication appears to be the cause of two phospholipase D enzyme. (Morris et al., 2019)

Outer Membrane Vesicles (OMVs): OMVs are secreted by many gram negative bacteria from the outer membrane. OMVs are known as delivery systems for bacterial effectors to the cells of the host. Generally, they are made of outer membrane and periplasmic proteins, DNA and/or RNA, LPS ad phospholipids. (C. R. Lee et al., 2017) For OMVs, interaction between pathogen and host cells can be done simultaneously without having any direct contact. In this process number of virulence factors come to the host cells. (C. R. Lee et al., 2017) Many strains of *A. baumannii* secrete OMVs containing numbers of various virulence factors. Likewise, phospholipases, OmpA, protease. *A. baumannii* generates OMVs that causes cytotoxicity. This OMVs transport bacterial effectors by lipid rafts after connecting to the host cells. *A. baumannii* with higher production level of OMVs are able to produce more virulence factors and are more cytotoxic. (C. R. Lee et al., 2017)

Metal Acquisition System: *A. baumannii* can modify its metabolic and nutritional requirements in order to adapt to the adverse host environment. *A. baumannii* needs nutritional metals to survive, much like all other species. Iron, zinc, manganese, copper, magnesium, and nickel are typically among these important metals, which work as co-factors for a number of essential biological activities. Additionally, *A. baumannii* exhibits a variety of tissue tropism and has evolved means of acquiring metal nutrients in varied host environments. (Mortensen & Skaar, 2013) The importance of iron levels to *A. baumannii* virulence is demonstrated by the way that *A. baumannii* responds to iron deficiency by altering the expression of numerous expected iron-related genes as well as genes involved in numerous functions like respiration, biofilm formation, and motility, i.e some strains make use of the iron-dependent repressor ferric uptake regulator (Fur). According to one study, iron deficiency enhances the production of PLCs, which in turn boosts *A. baumannii*'s hemolytic activity. (C. R. Lee et al., 2017) According to these observations, iron acquisition processes are essential for *A. baumannii* pathogenicity. (C. R. Lee et al., 2017) By chelating metals like zinc (Zn^{2+} and Zn) and manganese (Mn^{2+} and Mn) through the host, the innate immunological metal-chelating protein calprotectin prevents the growth of bacteria. (C. R. Lee et al., 2017) In contrast, *A. baumannii* can still spread illness when this nutritional immune protein is present in vivo. (C. R. Lee et al., 2017) The zinc acquisition system (ZnuABC) used by *A. baumannii* to overcome zinc limitation is up-regulated in Zn-limiting circumstances, and the znuBmutant strain experiences Zn famine at greater Zn concentrations than the wild-type strain. (C. R. Lee et al., 2017) ZnuB plays a role in the pathogenesis of lung infections caused by *A. baumannii*. (C. R. Lee et al., 2017)

Protein Secretion System: *A. baumannii* has a number of protein secretion mechanisms. (C. R. Lee et al., 2017) Type II secretion system (T2SS) is the most recent *A. baumannii* secretion system to be described. Which is employed when infecting eukaryotic hosts or that kill rival bacteria. (C.

R. Lee et al., 2017) Notably, a study revealed that numerous MDR *A. baumannii* strains possess a large, self-transmissible plasmid that harbors negative regulators for T6SS. (C. R. Lee et al., 2017)

Penicillin Binding Protein (PBP): PBP7/8 produced by the pbpG gene, which is frequently involved in b-lactam antibiotic resistance, is a virulence factor in *A. baumannii*. The loss of PBP7/8 may have an impact on peptidoglycan structure, which may impact vulnerability to host defense mechanisms, according to an analysis of bacterial morphology involving electron microscopy. (C. R. Lee et al., 2017)

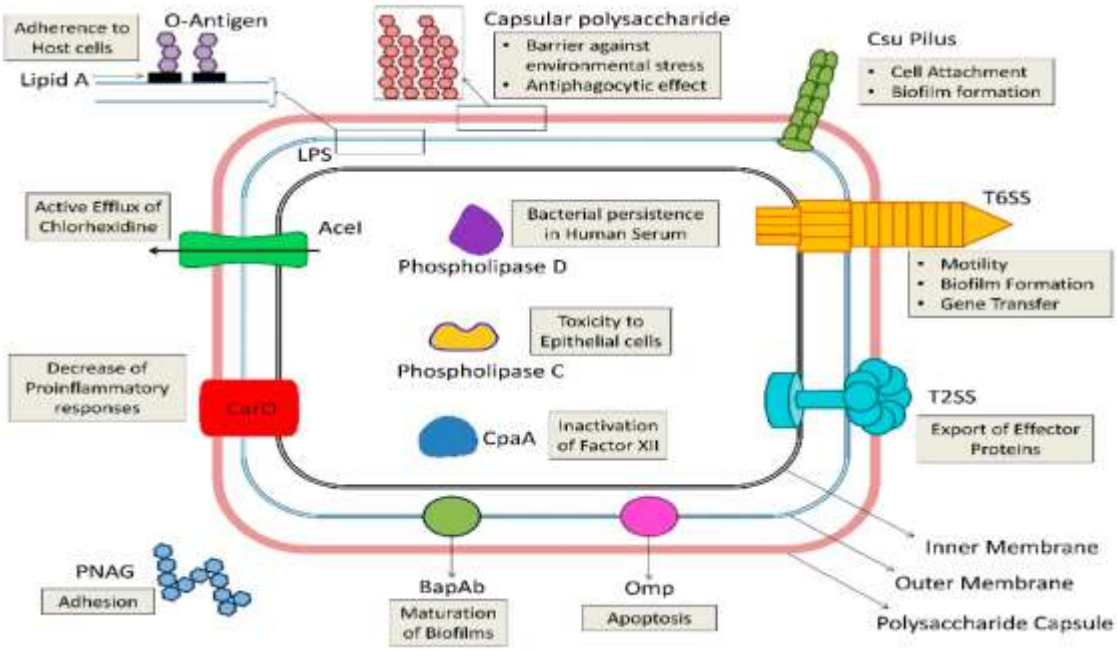


Figure 2: Diagrammatic representation of *Acinetobacter baumannii* virulence factors (Moubareck & Halat, 2020c)

2.5 Antibiotic Resistance Mechanisms

Because of its remarkable ability to develop antibiotic resistance, *Acinetobacter baumannii* has come to be known as one of the most prevalent infection causing bacteria in present-day healthcare. A number of strains of *A. baumannii* are very resistant to the majority of therapeutically accessible antibiotics (Lin and Lan, 2014). *A. baumannii* belongs to ESKAPE bacterial group, which pose a worldwide threat to humans as well as as a medical therapeutic challenge for dynamically increasing their resistance to multiple antibiotic drugs (Tacconelli et al., 2018).

A. baumannii uses several kinds of resistance mechanisms, such as β-lactamases, aminoglycosides-modifying enzymes, use of efflux pumps, defects in permeability, tetracycline resistance and target site modifications. The antibiotic classes that is still usable to treat *A. baumannii* infections in clinical settings has continuously decreased due to the development of many resistance mechanisms in *A. baumannii*. (Lee et al., 2017)

Beta-Lactamases:

Since the discovery of the first β-lactam antibiotic (penicillin), they have been used extensively in clinical practice to treat a variety of bacterial infections; β-lactam antibiotics are the preferred antibacterial medication. The peptidoglycan found in the cell walls of bacteria and fungi is the target of β-lactam antibiotic action, which inhibits bacterial growth or induces bacterial rupture. However, to break down β-lactam antibiotics enzymatically, some strains of *A. baumanii* bacteria can generate an enzyme called β-lactamase, which is the most common form of drug resistance. (Wu et al., 2023). Beta-Lactamases are classified into four categories based on variations in the hydrolytic technique and sequence motifs and homology and all four of these classes have been identified in *A. baumannii.*

1. Ambler class A enzymes: The primary enzymatic source of both innate and acquired resistance to β-lactams, particularly in *Acinetobacter baumannii*, is serine β-lactamases of molecular class A. This includes he extended-spectrum β-lactamase (ESBL) which are enzymes mediated by plasmid that causes hydrolysis and inactivation of broad spectrum β-Lactams antimicrobials, i.e third-generation cephalosporins, penicillins, aztreonam and carbapenems. This is causing a worldwide AMR problem mainly due to the production of the three main Ambler class A enzymes CTX-M, TEM and SHV β-lactamases. (Yousefi et al., 2021) Furthermore, the usage of antibiotics promotes the evolution and development of these enzymes' increased drug resistance.
2. Amber class B enzymes: These β-lactamases are known as metallo-β-lactamases (MBLs) that need the assistance of zinc or other heavy metal to perform catalysis. MBLs catalyze the hydrolysis of nearly all β-lactam antibiotics, including carbapenems, excluding monobactams, due to a wide substrate spectrum. *A. baumannii* has a range of class B β-lactamases found in it. The main Amber class B enzymes are NDM, IMP and VIM. The MBL gene may spread quickly with the help of plasmid, and the spread of NDM-1 has a direct relation to drug resistance in *Acinetobacter baumannii*. (Lee et al., 2017) NDM-1

has also been recovered from bacteria found in wound and bloodstream infections around the world. Due to its ability to hydrolyze a number of β-lactam antibiotics such as carbapenems, which are considered last choice antibiotics to treat of infections caused by resistant bacterial strains, it has become a significant source of worry for the clinicians, limiting treatment options. (Agarwal et al., 2018)

3. Amber class C enzymes: Cephalosporin antibiotic resistance is brought on by acinetobacter-derived cephalosporinases (ADCs). ADC overexpression results in drug resistance caused by ADC. Its overexpression is controlled by ISAba1 insertion making *A. baumannii* more resistant to extended-spectrum cephalosporins. Cefepime and carbapenems appear not be affected and remain stable against these enzymes. (Moubareck & Halat, 2020b) Because beta-lactamase inhibitors and beta-lactams have chemical similarities, *Acinetobacter baumannii* can easily acquire drug resistance to them. (Wu et al., 2023)
4. Amber class D enzymes: Oxacillinase (OXA), a D-type beta-lactamase, is connected to carbapenem resistance. The presence of oxacillinase, a member of the class D Ambler beta-lactamases, is the main cause of carbapenem resistance. More than 400 OXA enzymes that are encoded by genes located on chromosomes or plasmids have been described thus far. The OXA-51, OXA-24, OXA-23, OXA-58, and OXA-48 subgroups of carbapenem-hydrolyzing OXAs are particularly common in *A.baumannii*. While OXA-51 is intrinsic to *Acinetobacter baumannii*, OXA-23, OXA-24, OXA-48, and OXA-58 are acquired carbapenemases. (Moubareck & Halat, 2020b)

Treatment options have become more difficult due to the rapid rise of carbapenem-resistant *Acinetobacter Baumannii* (CRAB) prevalence in numerous nations and areas. (Kyriakidis et al., 2021) B-type and D-type play a major role in mediating carbapenem resistance. (Nguyen & Joshi, 2021)

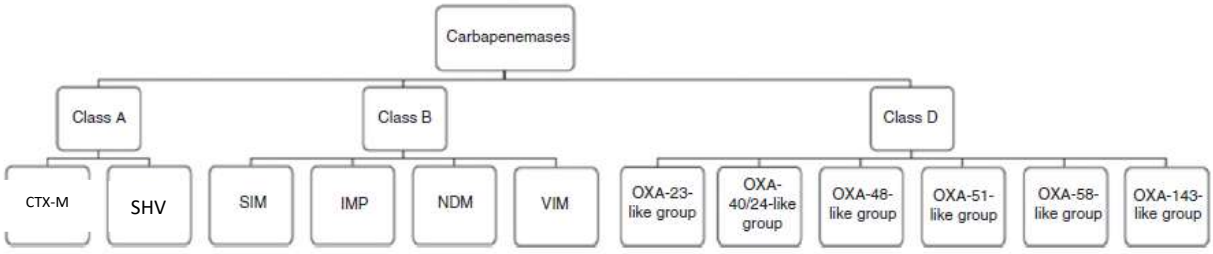


Figure 3: **Relevant carbapenemases examples that occur among Acinetobacter baumannii (adapted from Nguyen & Joshi, 2021).**

Efflux Pumps:

Higher efflux pump expression and beta-lactamases work simultaneously to increase antibiotic resistance (Cecchini et al., 2018). Cephalosporin and carbapenem resistance in *A. baumannii* is linked to overexpression of the AdeABC efflux pump (Huang et al., 2008; Hawkey et al., 2018; Magnet et al., 2001). AdeA is a protein that causes membrane fusion, AdeC is an outer membrane protein, and AdeB is a component that expels antibiotics from the cell (Vila et al., 2007). The the efflux pump's expression is controlled by a system called AdeRS. When the adeRS operon has point mutations it can cause the pump to express more frequently, which in turn results in antibiotic resistance (Marchand et al., 2004; Leus et al., 2018). AdeIJK and AbeM are some more efflux pumps have been demonstrated to play a role in imipenem and cephalosporin resistance (Hou et al., 2012; Yoon et al., 2015).

Permeability defects:

The permeability of an envelope can alter, affecting antibiotic resistance. For instance, porins play a crucial role in the pathogenicity of *A. baumannii* by creating channels that permit the transit of molecules through the outer membrane. Porins have an impact on membrane permeability, which makes them vital components in the resistance mechanism. In *A. baumannii*, reduced expression of a few porins is linked to carbapenem resistance. Increased imipenem resistance occurs when *A. baumannii* loses Omp29, which produces OXA-51- or OXA-23-like carbapenemases. (Moubareck & Halat, 2020b)

Aminoglycoside-modifying enzymes:

The primary method through which *A. baumannii* becomes resistant to aminoglycosides is through enzymes that modify aminoglycosides. Among the enzymes that modify aminoglycosides are acetyltransferases, adenyltransferases, and phosphotransferases. (Lee et al., 2017) *A. baumannii* can develop resistance to aminoglycosides (AG) in three different ways, per MicroBIGG-E. AMEs that reduce AG binding ability, 16S rRNA methyltransferases that change the target site, and aminoglycoside-modifying enzymes and restricted AG uptake due to impermeability or excessive efflux pump activity. (Kyriakidis et al., 2021)

AGs are inhibitors of protein synthesis that work by interfering with the elongation of peptide at the 30S ribosomal subunit after passing through the bacterial cell wall. Different ways like Integrons, transposons, and conjugated factors can all be used to convey genes that give resistance. (Kyriakidis et al., 2021) AG resistance genes can be transmitted at the cellular level as well as at the molecular level using transferable or conjugative plasmids, natural transformation, or transduction (Garneau-Tsodikova & Labby, 2016).

Tetracycline resistance:

Tetracycline antibiotics work by attaching to the 30S subunit of the ribosome and consequently prevent translation from beginning, preventing the production of proteins (Chukwudi, 2016). Three key pathways have been identified as the causes of tetracycline antibiotic resistance: (i) ATP-dependent efflux, (ii) inactivation of tetracycline by enzymes, and (iii) ribosomal protection proteins (RPPs) (Warburton et al., 2016). Tetracycline resistance in *A. baumannii* is brought on by two distinct types of energy-intensive efflux pumps- nonspecific, constitutive pumps called RND pumps and Tet efflux pumps

Alteration of target sites:

A. baumannii may develop antibiotic resistance as a result of changes to the drugs' target sites. Imipenem resistance is caused when mutated PBPs are overexpressed in the absence of other known resistance mechanisms. Multiple research studies have demonstrated that LPS alterations or deletion reduce *A. baumannii's* resistance to a number of clinically significant antibiotics, including colistin. (Lee et al., 2017)

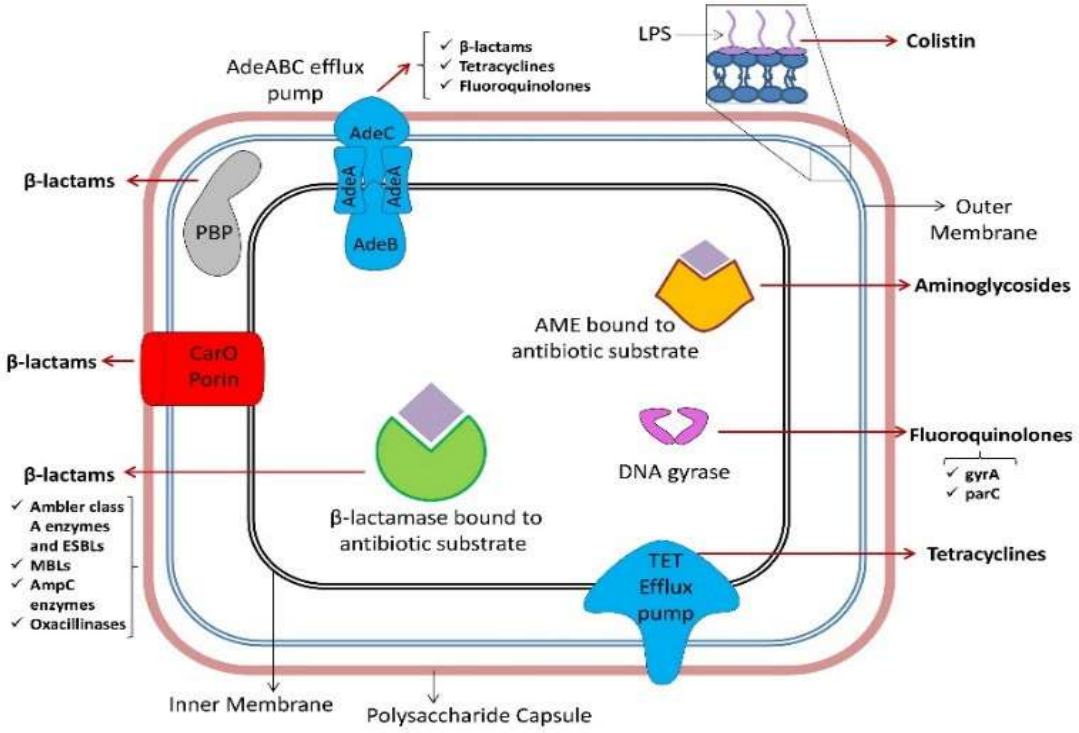


Figure 4: Acinetobacter baumannii resistance mechanisms to antibiotics/ antimicrobial agents (Moubareck & Halat, 2020)

2.6 Hospital wastewater as a potential reservoir of MDR *A. baumannii*:

Hospital wastewater are classified as a unique category due to their extreme hazard and toxic nature. It is a highly complex mixture of antibiotic compounds, dissolved medicines and bactericides, disinfectants, metabolised medications, and sensitive and resistant bacteria from hospitalised patients are all present in these kinds of wastewater. (Emmanuel et al., 2005, Lépesová et al., 2020) Antibiotic resistant pathogens may transfer from the hospital and spread into the sewage system. When hospital effluents are dumped straight into the sewer network without first receiving treatment, the problem may get worse (Lépesová et al., 2020) as such genes or pathogens can disseminate into the open environment. On top of that, multidrug-resistant (MDR) genes could be found in the excretion and microorganisms of patients (Galvin et al., 2010; Chagas et al., 2011). Thus, hospital wastewater is thought to be one of the main reservoirs for antibiotic resistance because they create a system where antibiotic resistance genes can be shared. The number of gram-negative bacteria with multiple bla genes such as bla-NDM, TEM, CTX-M, OXA48 and bla-SHV in clinical wastages is increasing at an alarming rate (Chagas et al., 2011; Zhang et al., 2012). There are certain pathogenic microbes that exhibit longevity in their lifespan when residing in aquatic environments, where they can transmit diseases and acquired antibiotic resistance genes (ARGs) from environmental reservoirs (Perron et al., 2008). Hospital wastewater is one of the largest contributors to the environmental burdens of antibiotics and subsequently antibiotic resistance. Antibiotic resistant genes that are available in an open environment may serve as a reservoir and can be transmitted horizontally to humans who come in contact with bacteria, leading to the development of antibiotic resistance (Khan et al., 2013). Moreover, even trace amounts of antibiotics present in wastewater act as signalling molecules and regulatory medium causing the induction of horizontal gene transfer, mutagenesis, and bacterial repair response and even mutation of resistant bacterial strains. (Lépesová et al., 2020)

According to Gordon et al. (2017), the hospital aqueous environment i.e drinking water, faucets, washbasin surfaces, and wastewater drainage points like drains can be a reservoir for nosocomial drug-resistant *A. baumannii*. Also according to Higgins et al. (2018), hospital wastewater is named as a major source of clinically important *A. baumannii*; in Brazil, China, and Croatia, viable multi-drug resistance (MDR) *A. baumannii* was discovered in untreated hospital wastewater while Croatian authorities recently described the spread of clinically significant viable *A. baumannii* from hospital effluent and municipal sewage into rivers.

2.7 Knowledge Gap in Existing Literature

While there are numerous studies and meta-analyses of *A.baumannii* from hospital settings such as nosocomial infections and clinical samples, information about *A. baumannii* in environmental samples like wastewater and community tap water is so far rare. To fill the literature gap, our

research focused on isolating *A. baumannii* from environmental samples of hospital wastewater and community tapwater to see if there is a connection or pathway to spread in Dhaka City. This is a case that has had very little research done in our country. Moreover the presence of ESBL Extended-spectrum Beta-lactamase (ESBL) encoding and Carbapenem-resistant A. baumannii in hospital wastewater was investigated in numerous researches but resistance to ESBLs and carbapenems has been scarely researched about bacteria isolated from community tap water and the existing knowledge is still incomplete and requires further research. To understand the total picture of antibiotic resistance pattern, it is not enough to do clinical survey, rather we must look into environmental isolates in order to see whether there is dissemination into communities which might cause diseases.

2.8 Novelty of our Study

Despite being common in the environment, over fifty percent of *Acinetobacter baumannii* have been isolated from humans. *A. baumannii* was mostly isolated from hospitals and, less commonly, from the environment outside of hospitals, although it is now more frequently detected from locations where human waste is evident. These locations contain a range of municipal wastewater types, including home, industrial, hospital, and stormwater. The primary source of *A. baumannii* that is clinically important is thought to be hospital effluent. (Hubeny et al., 2022) Sewage is a complex mix of elements from many faecal origins. Incorrectly handled hospital sewage may contain microorganisms and traces of antibiotics that might cause acute infections or be the source and transmission of resistance via HGT of transferrable resistance genes. (Zhang et al., 2013) Like this, the hospital may become a hotspot of spread of the MDR *A. baumannii* bacteria. Thus, the transfer and dispersion of hospital pathogenic organisms to the surroundings has been a major public health concern with a lack of research done in our country.

2.9 Aims, Objectives and Hypothesis

Our hypothesis is that hospital wastewater can serve as a reservoir for MDR *A. baumannii* and untreated wastewater discharge causes such pathogens to disseminte and spread to community tap water. Our choice of locations represents a reflection of our desire to help address issues that are prevalent in our immediate local environment as well as worrying trends related to challenges in treating infections brought on by the spread of resistant bacterial strains within communities. Therefore our study's aim was to explore the prevalence of antibiotic resistant *A. baumannii* isolated from hospital wastewater and to determine their spread to adjacent community tap water by employing conventional, standard and molecular microbiology techniques.

CHAPTER 3: METHODOLOGY

3.1 Sample Collection Site

For our study, we collected our subject of research from two sampling points- hospital effluents wastewater and neighboring household tap water. During the time span of March 2023 to June 2023, samples were collected every month from 3 different hospitals' sewage lines and four households surrounding each hospital. The hospitals selected for our research were Dhaka Shishu (Children) Hospital, National Institute of Cancer Research & Hospital (NICRH), and DNCC Dedicated Covid-19 Hospital. For the community tap water sample, 4 households were selected at random at a 300m radius distance from each hospital and samples were collected from the same households each month.

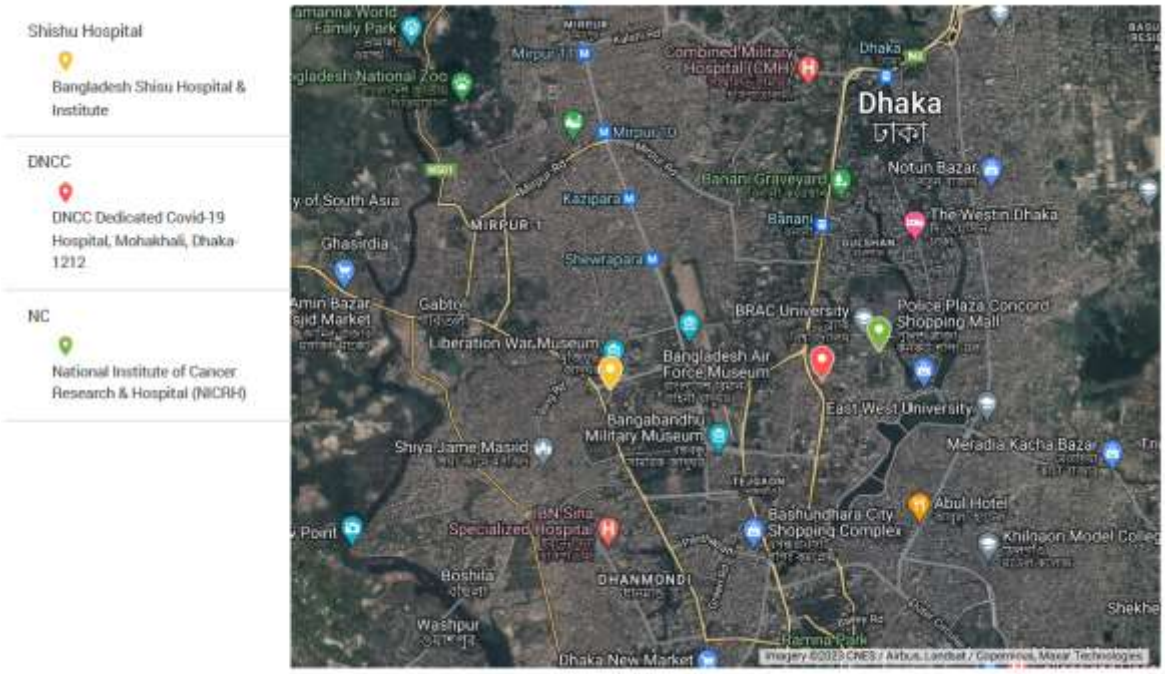


Figure 5: Sample collection sites of our study

3.2 Sample Collection Procedure and Transportation

The sewage water was collected in 50ml falcon tubes while household tap water was collected in 600ml sample collecting bottles. Both the bottles and falcon tubes were autoclaved at 121°C for 15 minutes and labelled, before taking them to the sample site, to maintain sterility. These sample containers were transported to and from the laboratory in an ice box to further decrease any chance

of contamination and preserve the sample's original state. All aseptic conditions were maintained during collection- gloves and masks were worn by the person in charge of collecting the samples. While opening the lid of the falcon or bottle, making contact with the inside of the container was avoided, and the lid was securely screwed shut immediately after the sample was poured to avoid leakage(Maje et al., 2020) and exposure to surroundings. In case of the hospital wastewater, sample was collected in two falcon tubes- to keep one as backup. Then, the sample was transported to the Microbiology Lab of BRAC University within 2 hours of collection and processing was done within 6 hours in order to prevent death or proliferation of microbes in the sample. (Champa & Kabir, 2018)

Figure 6: Icebox used for sample transportation

3.3 Sample processing

The sample processing started as soon as the samples reached the lab as the materials and reagents needed for the process were all prepared previously. The materials used were:

- Sterile test tubes containing 9ml saline (autoclaved)
- Autoclaved membrane filtration apparatus
- Falcon tubes containing 40ml Buffered Peptone Water, BPW (autoclaved)
- 0.45um membrane filter paper
- Media for culture: Leeds, MacConkey, Nutrient Agar poured into glass plates

The flowchart below represents the total workflow:

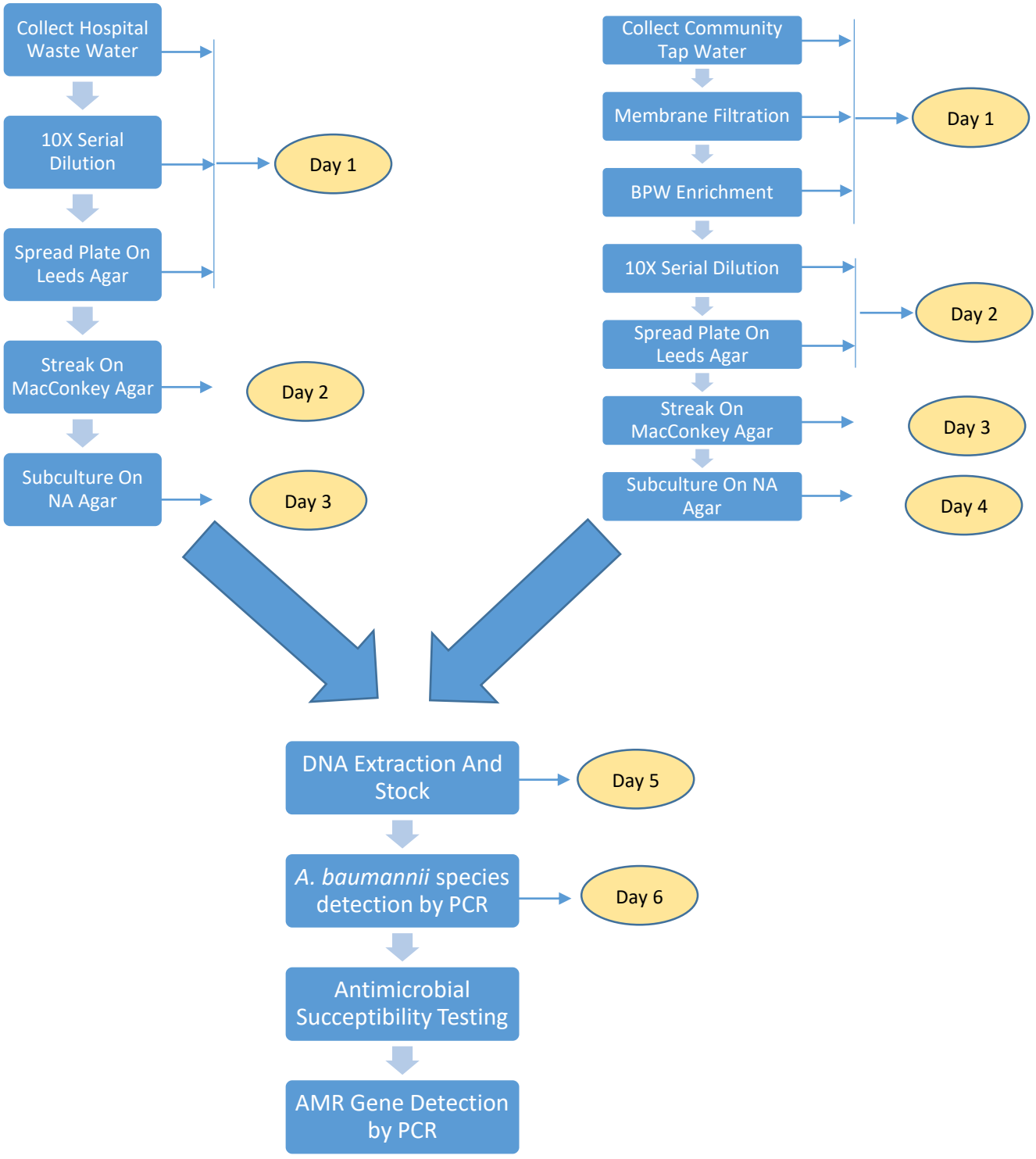


Figure 7: Sample Processing Workflow

3.3.1 Membrane Filtration, Serial Dilution and Spread Plating

When the samples arrived at the lab, the hospital sewage water was serially tenfold diluted by measuring out 1 mL of wastewater sample into a sterile test tube with 9 mL of sterilised 0.9% NaCl saline and diluting them from 10-1 to 10-8. (Mapipa et al., 2022) The dilution was performed inside a laminar air flow cabinet to maintain aseptic conditions and avoid cross contamination. Among the dilutions done, direct, 10^-3, 10^-4 and 10^-5 dilutions were selected for spread plating. 100ul of each dilution was spread over Leeds Acinetobacter Agar, LAM (HiMedia, India) before plates underwent a 24-hour incubation period at 44 °C. (Muzahid et al., 2023) A sterile glass spreader was used to evenly coat the inoculum on the agar media surfaces (Mapipa et al., 2022) and the plates were labelled appropriately before incubating.

On the other hand, for community tap water samples, membrane filtration was performed first. 100ml tap water from each community was poured into the filtration apparatus and filtered through 0.45um filter paper. The filter papers were then inoculated into separately labelled falcon tubes containing BPW (autoclaved), put inside a beaker and placed in a shaker incubator set at 37°C and 100 rpm for 24 hours. The purpose of this additional step is that BPW acts as an enrichment media for the successful isolation of colonies during solid media plating as tap water is speculated to have low microbial load which might not show up if the water is cultured directly. After 24 hours, growth inside the BPW culture was inspected by checking whether the liquid has turned turbid- indicating microbial proliferation. After this, the solution was serially diluted tenfold upto 10^-7 using 0.9% NaCl saline. Dilution was done inside a Laminar air flow cabinet. Among the dilutions done, 10^-3, 10^-4 and 10^-5 dilutions were selected for spread plating. Using the same process as the hospital wastewater, 100ml of each dilution was spread over Leeds Acinetobacter Agar (HiMedia, India) with a sterile glass spreader and labelled appropriately before plates underwent a 24-hour incubation period at 44 °C. (Muzahid et al., 2023)

3.3.2 Colony Morphology Based Selection and Solid Media Culture

At the end of the required incubation period, the plates were taken out of the incubator and observed to identify potential *A.baumannii* based on the colony morphology. *A. baumannii* grow as pink, circular, smooth mucoid colonies with mauve bases and mauve media colour on LAM. (Jawad et al., 1994) First, colony count of each plate (only for hospital wastewater plates) were taken to be converted to CFU/ml count. Then, around 3-4 such colonies from each LAM plate were selected to culture by streak plating on MacConkey media plates and incubated for upto 24 hours at 37°C. Standard streaking techniques were followed strictly in order to get isolated single colonies. After appropriate incubation, single colonies from the MacConkey plates were taken and

subcultured on NA media plates in order to get pure colonies of potential *A. baumannii*. A small amount of the colonies produced on NA media was taken with an inoculating needle and stabbed inside labelled vials containing T1N1 media and Soft Agar media to produce stock cultures to keep the organism viable to use if needed later. The vials were incubated at 37°C for 24-48 hours and then topped with sterile paraffin oil (for t1n1) and glycerol (for soft agar) to preserve them at room temperature.

3.4 DNA extraction

DNA extraction is a process used to separate DNA from other cell components like RNA, cell membrane, proteins (Gupta, 2019) and purifying it in order to use for other identification techniques. In our research, DNA is used to perform Polymerase Chain Reaction (PCR) for positive identification of our isolates as *A. baumannii*.

Boiling method was used to extract genomic DNA as it is overall the least time and reagent consuming method that produced good quality DNA. Proper steps and protocol were followed in order to get pure and adequate DNA suitable to be used for PCR. First, sterile microcentrifuge tubes (MCT), were labelled with our isolate IDs. 150ul Tris-EDTA (TE) buffer was aliquoted to each MCT. Then, with an inoculating loop, a loopful amount of bacterial colonies was taken from our NA subculture plates and dissolved in the TE buffer and subsequently vortexed to mix the cells throughout the buffer homogeneously. A dry heating block was used as the boiling source which was set for the temperature to rise to 100°C. Once risen, the vortexed MCTs were placed in the wells of the heating block and left to boil at 100°C for 15 minutes. After completion of this step, the MCTs were taken out of the block and put in a centrifuge machine and centrifuged at 14000 rpm for 6 minutes. After 6 minutes, the MCT was observed to see the formation of a pellet (containing cell debris and components) at the bottom and a clear liquid called supernatant containing the separated bacterial DNA. Subsequently, the supernatant was transferred to another labelled MCT and stored at -20°C until performing PCR.

3.5 Identification of *A. baumanii* using conventional Polymerase Chain Reaction and Agarose Gel Electrophoresis

Polymerase chain reaction (PCR) is a method used to amplify/ multiply a specific section of DNA very quickly into millions or billions of copies, which is then used for more in-depth analysis of the said amplified section of the DNA or to be used in identification of a particular organism. In PCR, first a section of the gene to be amplified is marked using short DNA fragments called

primers which are synthesized specifically. Then, multiple cycles of DNA synthesis are used to amplify that segment. (*Polymerase Chain Reaction (PCR)*, n.d.) In our study, in order to positively identify the presence of *A. baumannii* on a molecular level among our chosen isolates, PCR was performed based on the detection of *blaOXA-51* gene. (Falah et al., 2019) Conventional PCR was used. The primers used in our study are given in Table. 1

Gene/Primer Name	Primer Sequence	Amplicon Size (bp)	PCR conditions	Reference
blaOXA-51	F:TAATGCTTTGATCGGCCTTG R:TGGATTGCACTTCATCTTGG	353	Initial Denaturation: 94°C for 5 min Denaturation: 94°C for 30 sec Annealing: 55°C for 30 sec Elongation: 72°C for 30 sec Final Elongation: 72°C for 7 min	(Woodford et al., 2006)

Table 1: Primers used for molecular identification of *A.baumannii*

Sterilized PCR tubes and MCT were used during the process. Total PCR mixture was prepared in a 1.5ml MCT and 11ul reaction mixture was aliquoted to 0.2ml PCR tubes for each isolate, abd then DNA was added separately to each tube. Thus, the 13ul PCR mix comprised of 7.5ul PCR Master Mix, 2.5ul DNase/ RNase free water, 0.5ul of each set of blaOXA-51 primers (forward and reverse) and 2ul of our previously extracted DNA to be used as template. (Falah et al., 2019) After all the tubes were ready, they were placed inside Applied Bio-system (Thermo-Fisher) thermal cycler and the condition was set as follows: Initial Denaturation at 94°C for 5 min, Denaturation at 94°C for 30 sec, Annealing at 55°C for 30 sec, Elongation at 72°C for 45 sec and Final Extension at 72°C for 7 min. The machine was set to perform 30 cycles.

After PCR was completed, the products were removed and examined for band size using Agarose Gel Electrophoresis (AGE). AGE is a process to effectively separate, identify and purify DNA fragments of varying sizes from 0.5- to 25-kb. We used the horizontal setup. The amplified PCR products were run in 1.5% agarose gel submerged in TBE buffer (40 mM Tris, 20 mM boric acid, 1 mM EDTA, pH of 8.3) containing 4ul µg/100mL Ethidium Bromide dye. Each amplified PCR

product was carefully and slowly loaded into separate wells of the solidified gel. A 100bp DNA ladder was also used for band size comparison; a previous positive sample of *A. baumannii* was used as the positive control and a no-template control (nuclease free water) was used as the negative control. The gel was run at 110V and 500mA for 60 minutes for ideal separation of the DNA ladder. At the end of the run, the gels were visualised under an Ultraviolet Transilluminator (Cleaver Scientific). Isolates were accepted as positive for *A. baumannii* if a band was visible at the correct predicted size for blaOXA-51 (Falah et al., 2019), i.e 353 bp (between the 300 and 400 band of the ladder).

3.6 Antibiotic Susceptibility Test (AST)

Confirmed *A. baumannii* isolates were analyzed for antimicrobial susceptibility testing to find the resistance pattern towards different classes of antibiotics. The Kirby-Bauer disk diffusion method was used in accordance to the guidelines of the Clinical Laboratory Standards Institute (CLSI). All *A. baumannii* isolates were tested against antibiotic discs impregnated with for 11 different therapeutically relevant antibiotics (Obeidat et al., 2014): Gentamicin (10 μg) and Amikacin (30 μg) belonging to aminoglycosides class; Imipenem (10 μg) belonging to carbapenem class; Cefixime (5 μg) and Ceftazidime (30 μg) belonging to 3rd gen cephalosporins class; Cefepime (30 μg) belonging to 4th gen cephalosporins class; Levofloxacin (5 μg) belonging to Fluoroquinolones class; Erythromycin (15 μg) belonging to Macrolides class; Amoxicillin+clavulanic acid (30 μg) and Doxycycline (30 μg) from penicillin class; Aztreonam (30 μg) from monobactams class.

To perform the AST, Mueller–Hinton agar (MHA) was used. At first, the PCR confirmed isolates were subcultured on nutrient agar media and incubated for 24 hours at 37°C. From this fresh culture, a small amount was taken on an inoculating loop and dissolved in test tubes containing 5ml of sterilized 0.9% NaCl saline. The suspension was vortexed thoroughly and optimized to 0.5 McFarland turbidity standards. (Agarwal et al., 2018) Next, a sterile cotton swab was dipped into the suspension to soak it and then firmly rotated against the upper inside wall of the test tube to remove excess liquid. The entire MHA plate was streaked with the swab to create a lawn all over the agar surface. This was done 4 times by rotating the plate at 45 ° angle between each streaking until a sticky consistency was found between the agar and the swab. After lawning, the chosen antibiotic discs were applied using aseptic techniques- using forceps heat sterilized each time. Antimicrobial discs were placed equidistantly on the plate. The plates were incubated at 37°C for 18-24 hours. After that, inhibition zone diameters were measured. Values obtained were interpreted according to guidelines endorsed by the CLSI interpretive criteria and subjected to minimum inhibitory concentration (MIC) values in order to determine whether they fall under “Sensitive”, “Intermediate” or “Resistant”.

The table below shows the Sensitive, Intermediate and Resistant ranges of the antibiotics used for *A. baumannii*.

Name of the Antibiotics	Sensitive	Intermediate	Resistant
Gentamicin	≥15	13-14	≤12
Amikacin	≥17	15-16	≤14
Imipenem	≥22	19-21	≤18
Cefixime	≥19	16-18	≤15
Ceftazidime	≥18	15-17	≤14
Cefepime	≥18	15-17	≤14
Levofloxacin	≥19	16-18	≤15
Erythromycin	≥23	14-22	≤13
Amoxicillin+ Clauvlanic Acid	≥18	14-17	≤13
Doxycycline	≥13	10-12	≤9
Aztreonam	≥21	18-20	≤17

Table 2: List of antibiotics used for AST

3.7 Multidrug Resistance (AMR) Gene Detection:

After phenotypic testing via AST, isolates that showed most resistance to antibiotics were lastly screened for the presence of the following resistant genes: CTX-M, SHV, TEM and NDM-1, OXA-48 and IMP. Same PCR method was followed as before, with changes in reaction mixture amounts and PCR conditions. These are given in the table below:

Gene	Primer Sequence	Amplicon Size	PCR condition	Reference
blaCTX-M	F:ACGCTGTTGTTAGGAAGTG R:TTGAGGCTGGGTGAAGT	759 bp	Initial Denaturation- 94°C for 5 min Denaturation- 94°C for 30sec Annealing- 58°C for 30 sec Elongation- 72°C for 30 sec Final Elongation- 72°C for 7 min	(Yousefi et al., 2021b)
blaSHV	F:TACCATGAGCGATAACAGCG R:GATTTGCTGATTTCGCTCGG	450 bp	Initial Denaturation- 94°C for 5 min Denaturation- 94°C for 30sec Annealing- 58°C for 30 sec Elongation- 72°C for 30 sec Final Elongation- 72°C for 7 min	(Doosti et al., 2015)
blaTEM	F:ATAAAATTCTTGAAGACGAAA R:GACAGTTACCAATGCTTAATCA	1080 bp	Initial Denaturation- 95°C for 3 min Denaturation- 95°C for 30sec Annealing- 51°C for 30 sec Elongation- 72°C for 30 sec Final Elongation- 72°C for 7 min	(Y. Zhang et al., 2021)

blaNDM-1	F:GGTTTGGCGATCTGGTTTTC R:CGGAATGGCTCATCACGATC	621 bp	Initial Denaturation- 94°C for 5 min Denaturation- 94°C for 30sec Annealing- 58°C for 30 sec Elongation- 72°C for 30 sec Final Elongation- 72°C for 7 min	(Al-Sultani & Al-Taai, 2020)
blaIMP-1	F:GAAGGCGTTTATGTTCATAC R:GTATGTTTCAAGAGTGATGC	587 bp	Initial Denaturation- 95°C for 5 min Denaturation- 95°C for 50 sec Annealing- 57°C for 30 sec Elongation- 72°C for 40 sec Final Elongation - 72°C for 10 min	(Khosravi & Mihani, 2008)
blaOXA-48	F:GCTTGATCGCCCTCG ATT R:GATTTGCTCCGTGGC CGAAA	281 bp	Initial Denaturation- 95°C for 5 min Denaturation- 95°C for 30 sec Annealing- 57°C for 30 sec Elongation- 72°C for 40 sec Final Elongation - 72°C for 10 min	(Gurung et al., 2020)

Table 3: Primers used for detection of AMR genes

CHAPTER 4: RESULTS & OBSERVATION

4.1 Isolation of *Acinetobacter baumannii*

Within the study period of March 2023 to June 2023, a total of 37 samples were collected for our research, out of which 9 were from Hospital waste water and 28 were from adjacent community tapwater. Isolates were primarily selected for microbiological and molecular testing based on the colony morphology seen on LAM spread plates and MacConkey streak plates. In LAM plates, presumptive *Acinetobacter baumannii* was chosen by picking colonies that had light pink, circular, smooth, mucoid and convex morphology with mauve color diffused into the medium. 2-4 colonies with slightly differing morphologies were randomly chosen from each plate and presumed to be suspected *A. baumannii*. The suspected colonies from LAM were streak plated onto MacConkey media and further morphology-based *A. baumannii* was confirmed if the colonies showed a pale, almost transparent colour. The size of the colonies were very little and many single colonies were seen to grow.

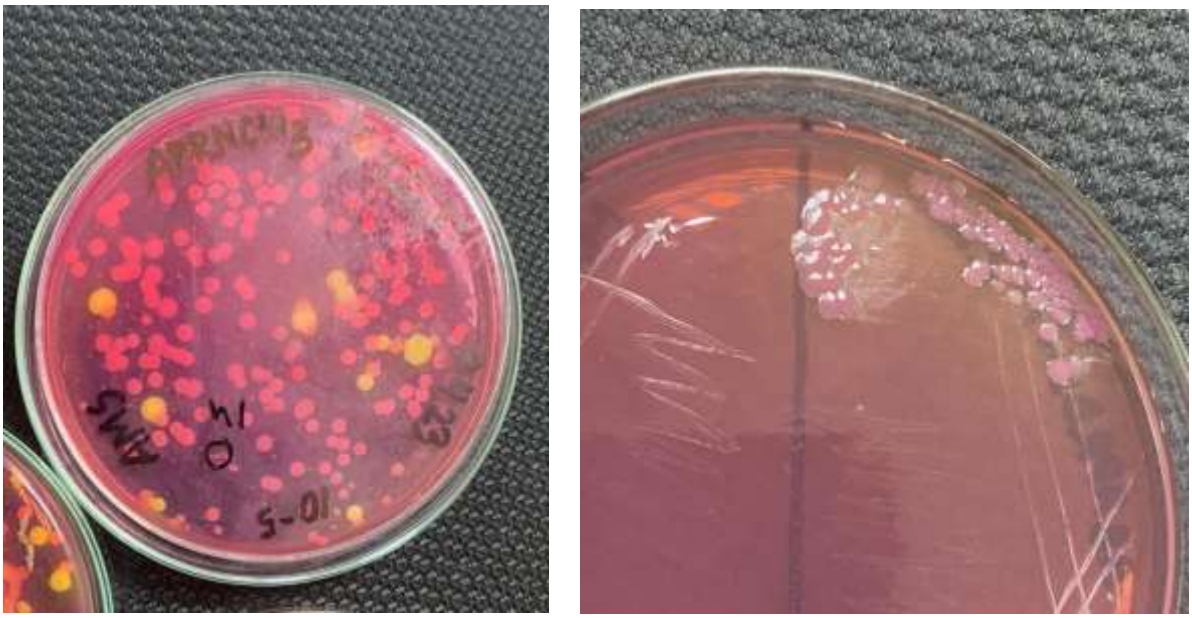
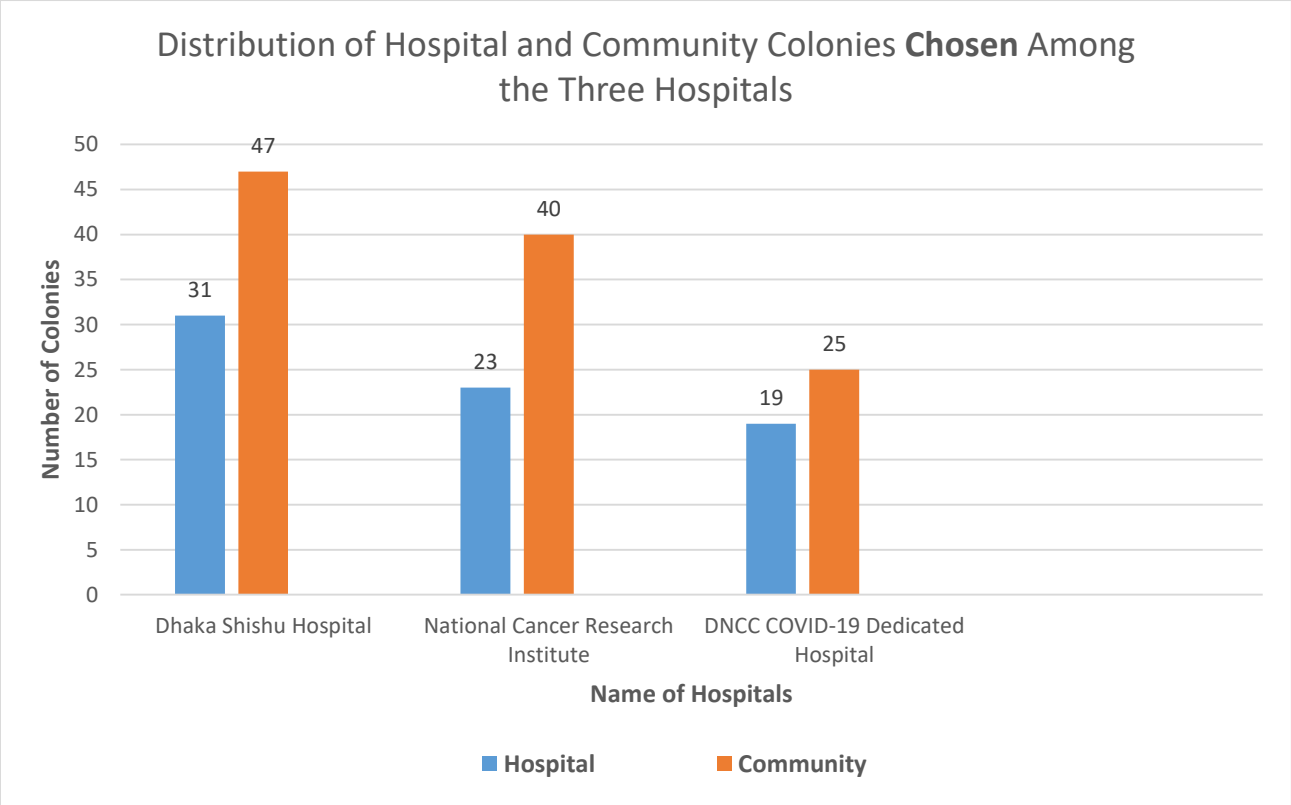


Figure 8: Suspected *A. baumannii* pink mucoid colony in LAM and pale pink colony in MacConkey media

4.2 Presumptive *A. baumannii* isolates chosen based on colony morphology:

A total of 185 such predicted *A. baumannii* colonies were chosen out of which 73 were from hospital wastewater source and 112 were from adjacent community tap water source. These isolates were subjected to PCR routinely in order to verify whether they were *A. baumannii* or not.

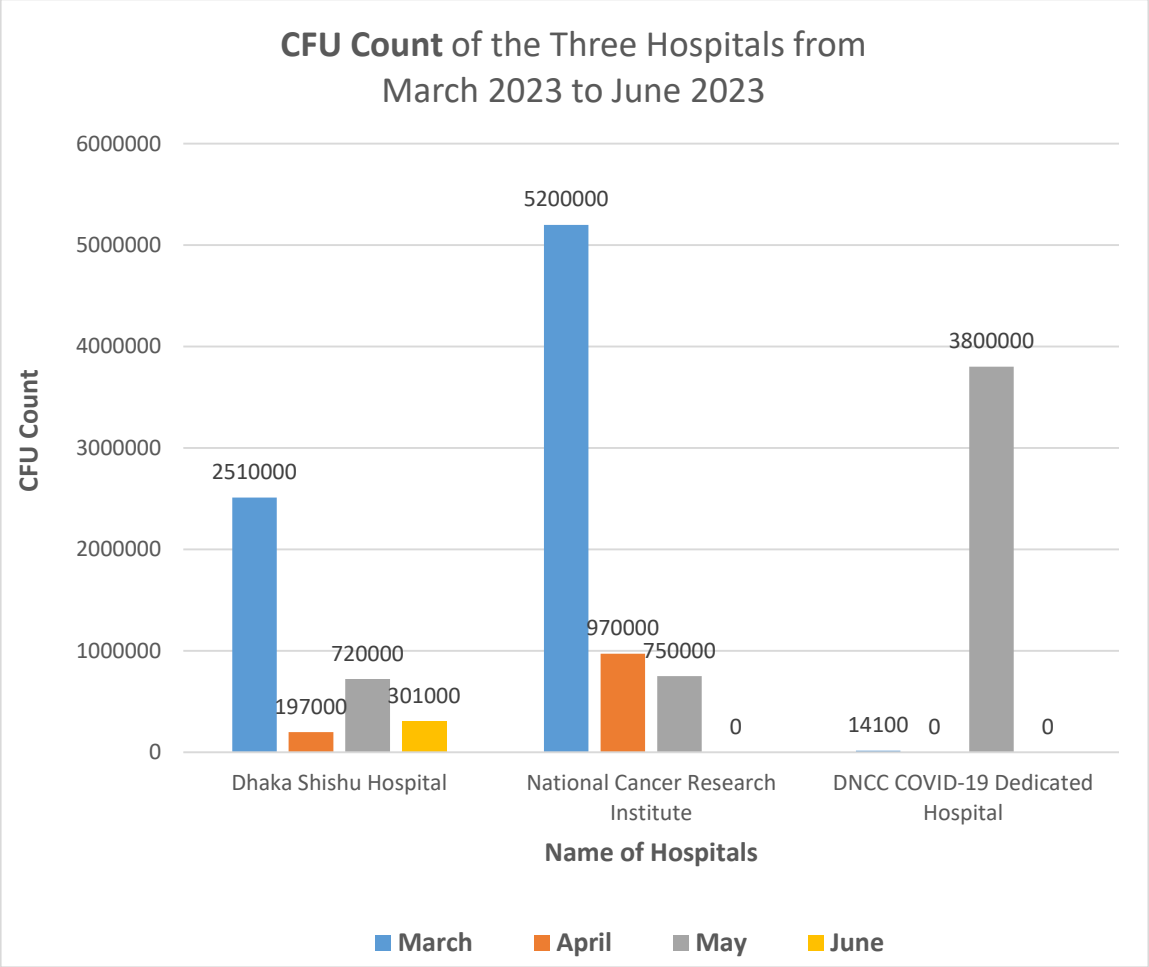
Among the chosen total 185 isolates, the graph below shows the number of isolates from each sample collection sites, i.e the three hospitals as well as the number of hospital wastewater isolates and community tap water isolates of each hospital. From Dhaka Shishu Hospital, 31 isolates were of wastewater source and 47 were from community tap water source. From National Cancer Research Institute, 23 isolates were of wastewater source and 40 were from community tap water source. And lastly, from DNCC COVID-19 Dedicated Hospital, 19 isolates were of wastewater source and 25 were from community tap water source.



Graph 1: Distribution of Hospital and Community presumptive *A. baumannii* Chosen among the Three Hospitals

4.3 Analysis of Colony Forming Unit (CFU)

Colony forming unit (CFU) counts were taken from the spread plates of hospital wastewater. The highest CFU count was obtained on March 2023 from National Cancer Research Institute, i.e 5.2 X 10^6. The lowest CFU count among all the samples collected was from March 2023 as well but from DNCC COVID-19 Dedicated Hospital, i.e 1.41 x 10^4. Overall, the highest average CFU count was from National Cancer Research Institute. The lowest was from DNCC COVID-19 Dedicated Hospital with the exception of the month of May when there was a drastic increase of CFU in this hospital compared to its other months.



Graph 2: CFU Count of the Three Hospitals from March 2023 to June 2023

4.4 Identification of *Acinetobacter baumannii* using PCR and Gel electrophoresis:

PCR and agarose gel electrophoresis was performed for species identification. The gel was observed over a UV transilluminator to observe the banding pattern and match it to the required band size. To detect *A. baumannii* positive isolates, band was seen at 353bp region along the 100bp DNA ladder used. The image below shows the positive isolates of National Cancer Research Institute for the month of April where 9 isolates of *A. baumannii* was confirmed as shown by the bands at 353bp level. Viability of the PCR and gel run was confirmed by observing the positive and negative control- positive control band was observed at 353bp and negative control band did not appear- verifying that the PCR and gel run was done successfully.

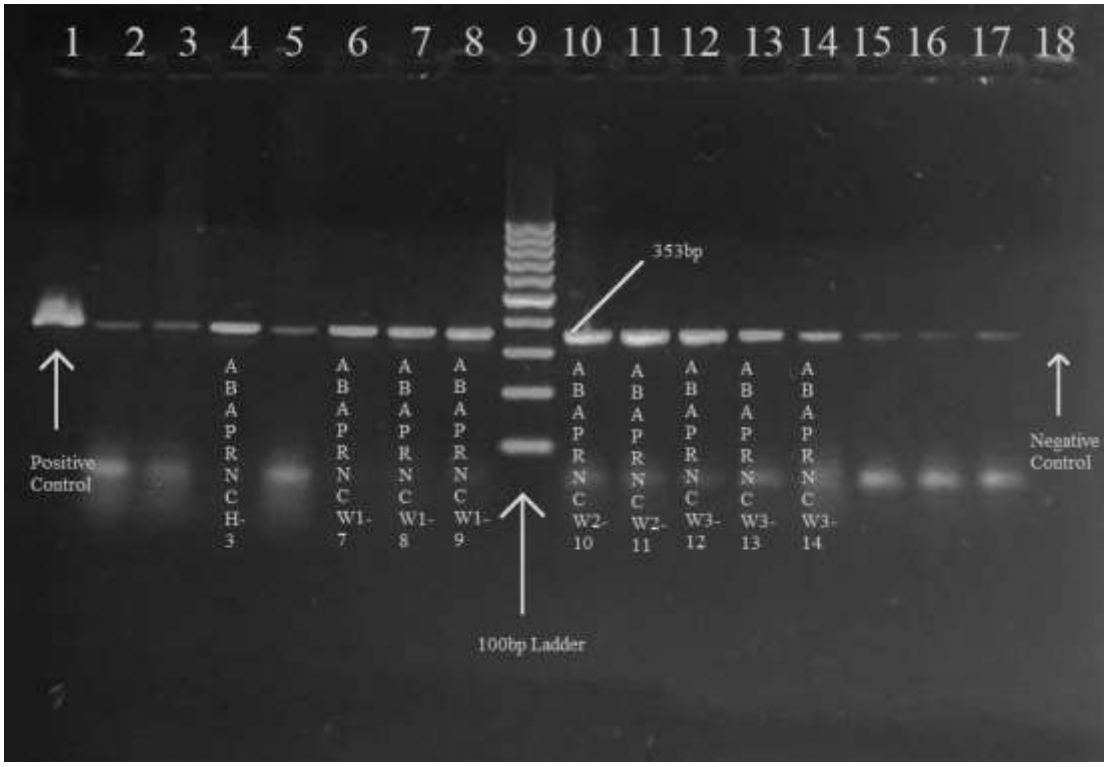
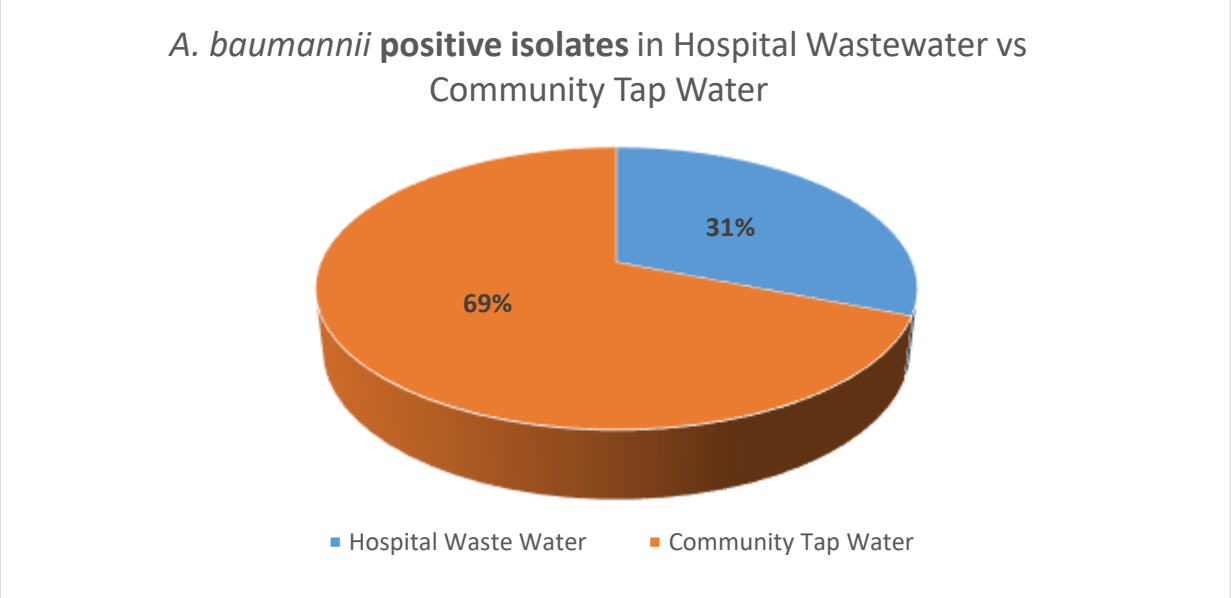


Figure 9: Gel visualization of *A. baumannii* species identification via PCR

4.5 Confirmed *A. baumannii* found in Hospital wastewater vs Community tapwater

From March 2023 to June 2023, total 145 isolates were undergone PCR and 100 of those isolates came out positive for *A. baumannii*, totaling 69% of our sample size. Among these 100, 31 isolates were from Hospital wastewater source and 69 isolates were from Community tap water source.

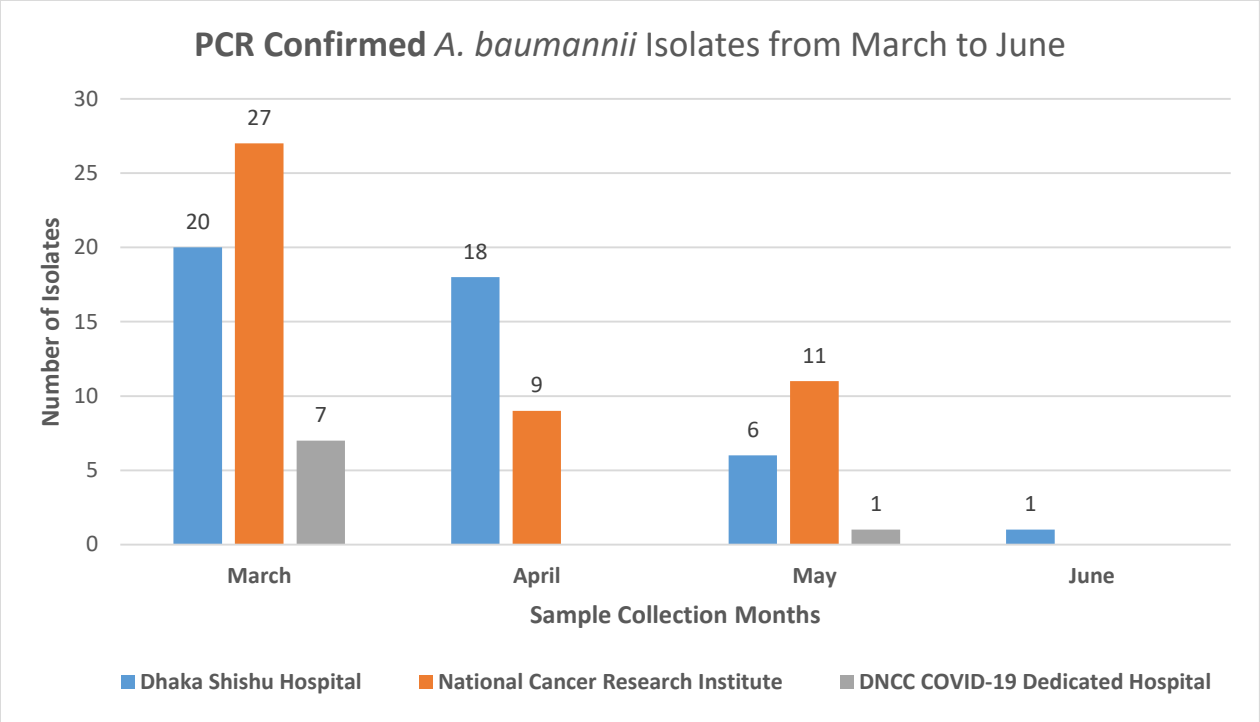


Graph 3: Positive *A. baumanni* found in Hospital wastewater Vs. Community tap water

4.6 Monthwise distribution of *A. baumannii* among the 3 hospitals

This study aimed to find the patterns of *Acinetobacter baumannii* with time, particularly from March 2023 to June 2023. The month wise distribution is illustrated below through the graph below.

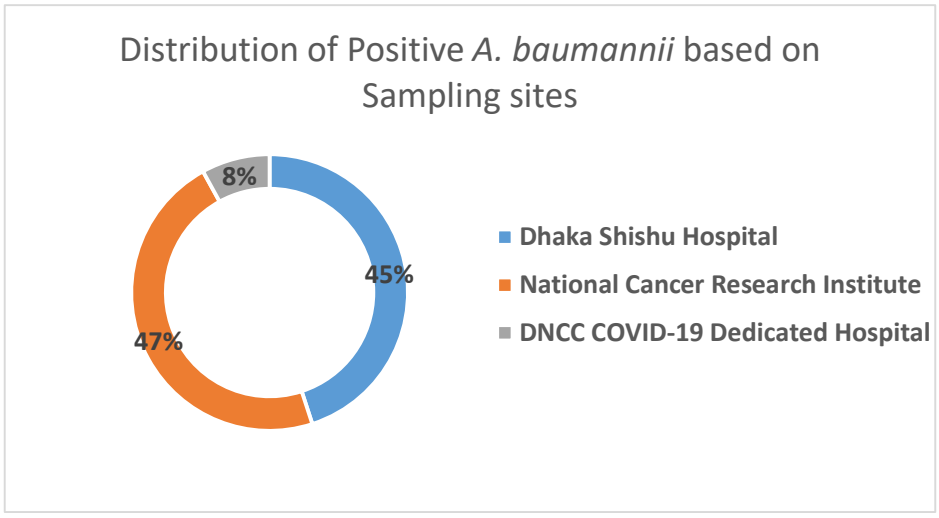
Overall, the number of positive *A. baumannii* decreased gradually from March to April to May to June. Highest number was found in March- totaling 54 PCR confirmed isolates. 27 confirmed isolates were found in April, 18 in May and only 1 in June. Hence, a downward relationship with time can be observed between the number of positive *A. baumannii* in the sampling sites. The highest number (27 isolates) of *A. baumannii* was found in March 2023 from National Cancer Research Institute, while the lowest was found in May 2023 and June 2023 from DNCC COVID-19 Dedicated Hospital and Dhaka Shishu Hospital respectively having 1 isolate each. Overall, it can also be seen that DNCC COVID-19 Dedicated Hospital has the lowest number of confirmed isolates in each month.



Graph 4: Month-wise distribution of positive *A. baumannii* among the 3 hospitals

4.7 Distribution of positive A. baumannii among the sampling sites

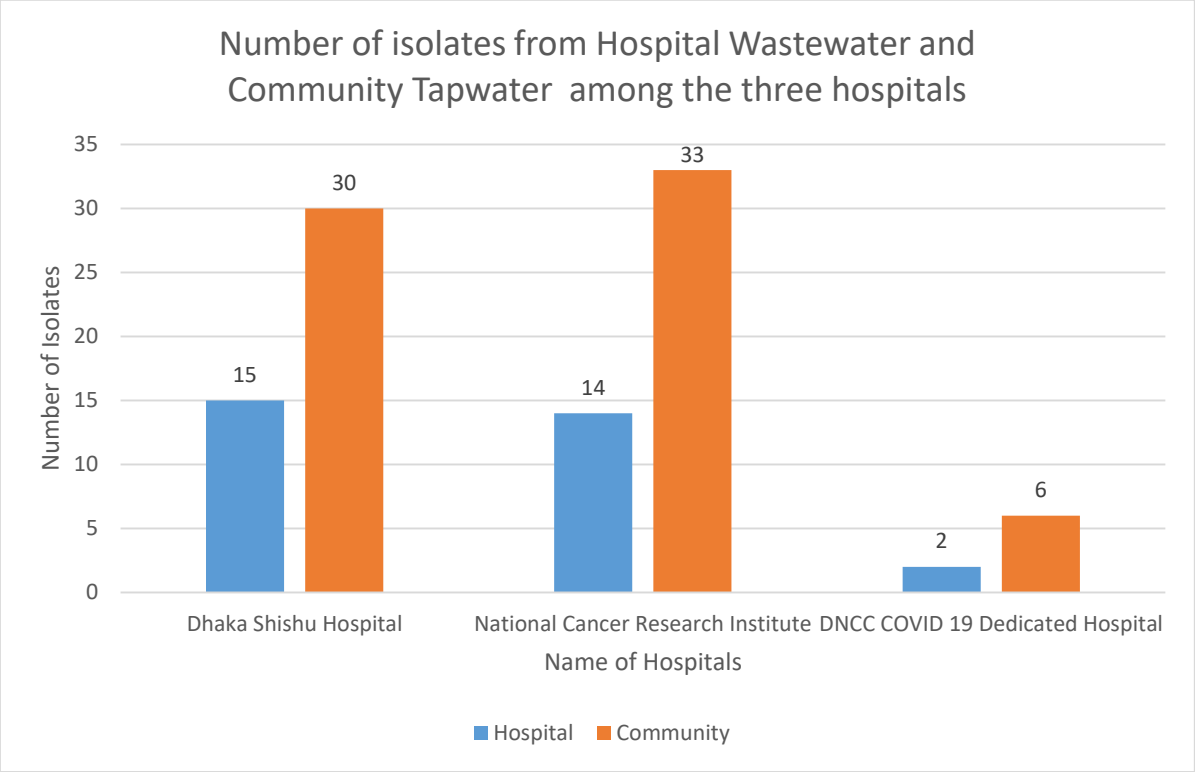
After analyzing the total data, it was found that 47% (47 out of 100) of the confirmed isolates were from the National Cancer Research Institute which was the highest among the 3 sampling sites. On the other hand, lowest number of isolates was observed from DNCC COVID-19 Dedicated Hospital which was 8% (8 out of 100). Lastly, Rates 45% (45 out of 100) was found from Dhaka Shishu Hospital which was almost as much as National Cancer Research Institute.



Graph 5: Distribution of Positive *A. baumannii* based on Sampling sites

4.8 Differentiation between the number of isolates retrieved from Hospital wastewater and Community tapwater of each hospital

Among the 45 confirmed isolates of Dhaka Shishu hospital, 15 isolates were from wastewater source and 30 isolates were from tap water source. Similarly, for National Cancer Research Institute, 14 out of 47 were from wastewater and the rest 33 were from tap water. In case of DNCC COVID-19 hospital, out of 8, 2 were from wastewater and 6 from tap water.



Graph 6: Positive isolates from Hospital Wastewater and Community Tap water among the three hospitals

4.9 Interpretation of Antibiotic Susceptibility Test Results:

After incubation for 18-24 hours, zone of inhibition around each antibiotic disc was measured for all 11 antibiotics used and the data was interpreted to be “Sensitive”, “Intermediate” or “Resistant” using the range as listed in Table-3. Satellite colonies were seen on some zones, which was also noted accordingly.

Figure 10: MHA plates after incubation for AST

4.10 Antibiotic resistance pattern of total *A. baumannii* isolates

From our data gathered, the most prominent resistance was seen against Cefixime, Ceftazidime and Azetreonam. All 52 isolates showed resistance against Cefixime (100%) and 50 isolates showed resistance against Ceftazidime (96%) – both of which are 3rd gen cephalosporins antibiotic class. In case of Azetreonam which is a Monobactam antibiotic, 84.6% isolates (44 out of 52) showed complete resistance while 13.5% isolates (7 out of 52) showed intermediate level resistance.

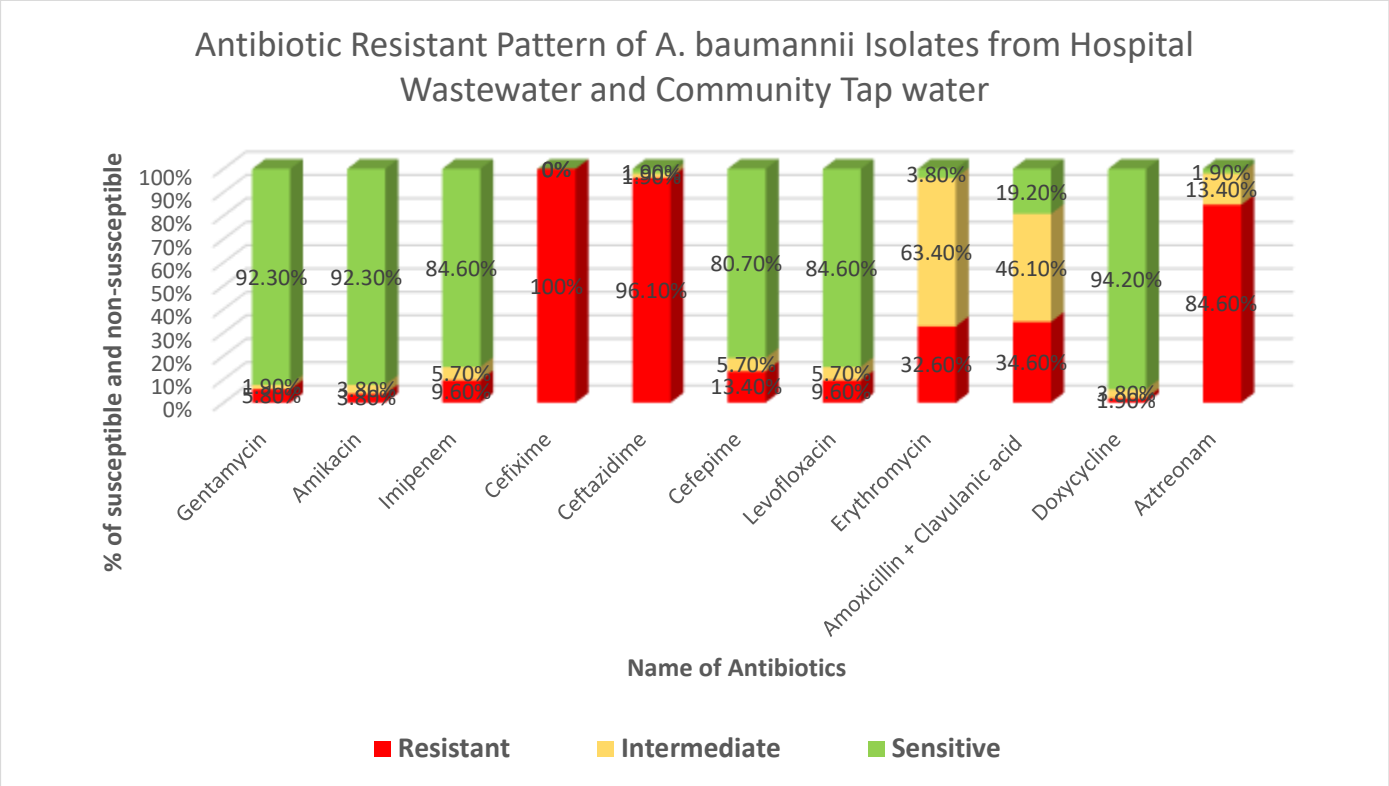
Next, isolates showed a high % of intermediate susceptibility against Erythromycin and Amoxicillin + Clavulanic Acid (AMC). For Erythromycin, 32.7% isolates (17 out of 52) were resistance and 63.5% (33 out of 52) were intermediate- making a 96% overall percentage susceptibility. Following a similar trend, 34.6% isolates (18 out of 52) were resistant and 46.2% (24 out of 52) isolates were intermediary against Amoxicillin + Clavulanic Acid totaling 80.8% percentage susceptibility.

Lastly, for the rest of the antibiotics, a higher percentage of sensitivity was seen, ranging from highest 94.2% isolates (49 out of 52) against Doxycycline to 80.7% isolates (42 out of 52) against Cefepime.

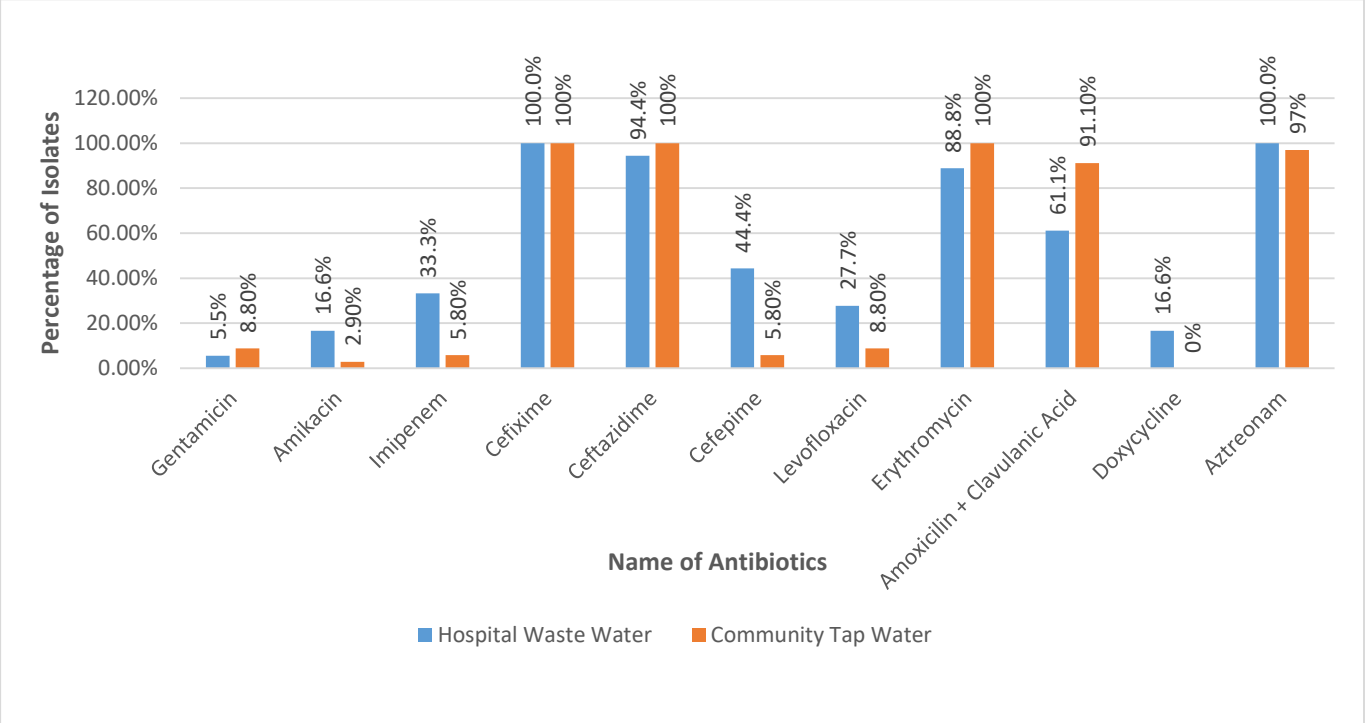
The Antibiotic resistant pattern data of all isolates has been illustrated in the following table and bar chart:

Antibiotic	% Resistant	% Intermediate	% Sensitive
Gentamycin(GEN)	5.8%	1.9%	92.3%
Amikacin(AK)	3.8%	3.8%	92.3%
Imipenem(IPM)	9.6%	5.7%	84.6%
Cefixime (CFM)	100%	0%	0%
Ceftazidime(CAZ)	96.1%	1.9%	1.9%
Cefepime(CPM)	13.4%	5.7%	80.7%
Levofloxacin(LE)	9.6%	5.7%	84.6%
Erythromycin(E)	32.6%	63.4%	3.8%
Amoxicillin + Clavulanic Acid (AMC)	34.6%	46.1%	19.2%
Doxycilin(DO)	1.9%	3.8%	94.2%
Aztreonam(AT)	84.6%	13.4%	1.9%

Table 4: Antibiotic resistance pattern of total *A. baumannii* isolates



Graph 7: Antibiotic Resistant Pattern of total *A. baumannii* Isolates



Graph 8: Comparison of % of Susceptibile Isolates between Hospital Wastewater and Community Tap Water

4.11 Results of PCRs done to detect various AMR genes in *A. baumannii*

After phenotypically testing the positive *A. baumannii* by AST, the isolates that showed most resistance to the antibiotics were chosen for further genotypic assessment by AMR gene-specific PCR. The genes that were chosen are given in the flow chart below. Out of 52 isolates, a resistance gene- namely NDM-1 was detected in 5 isolates. The detection for the other AMR genes, namely CTX-M, SVH, TEM, OXA-48 and IMP turned out negative- i.e these genes were observed to be not present in the chosen isolates.

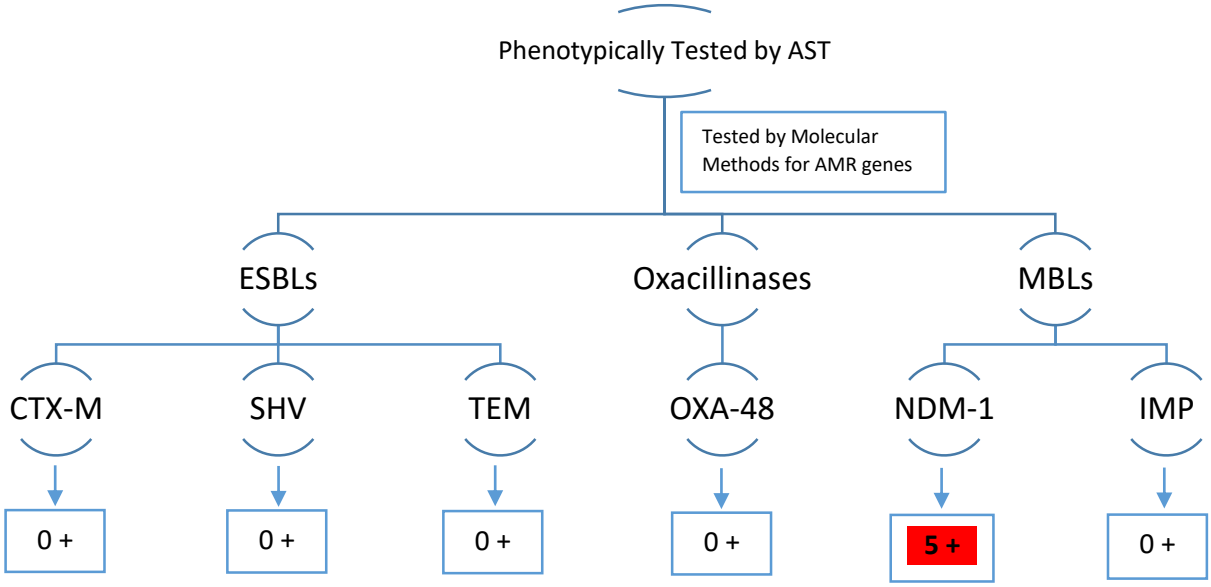


Figure 11: Results of PCRs done to detect various AMR genes in *A. baumannii*

Out of the 52 isolates tested for the presence of AMR gene, 5 showed the presence of NDM-1, which were 3 isolates from the March sample of Dhaka Shishu Hospital wastewater source; 1 isolate from April Dhaka Shishu Hospital wastewater source and 1 isolate from May National Cancer Reasearch Institute community tapwater source.

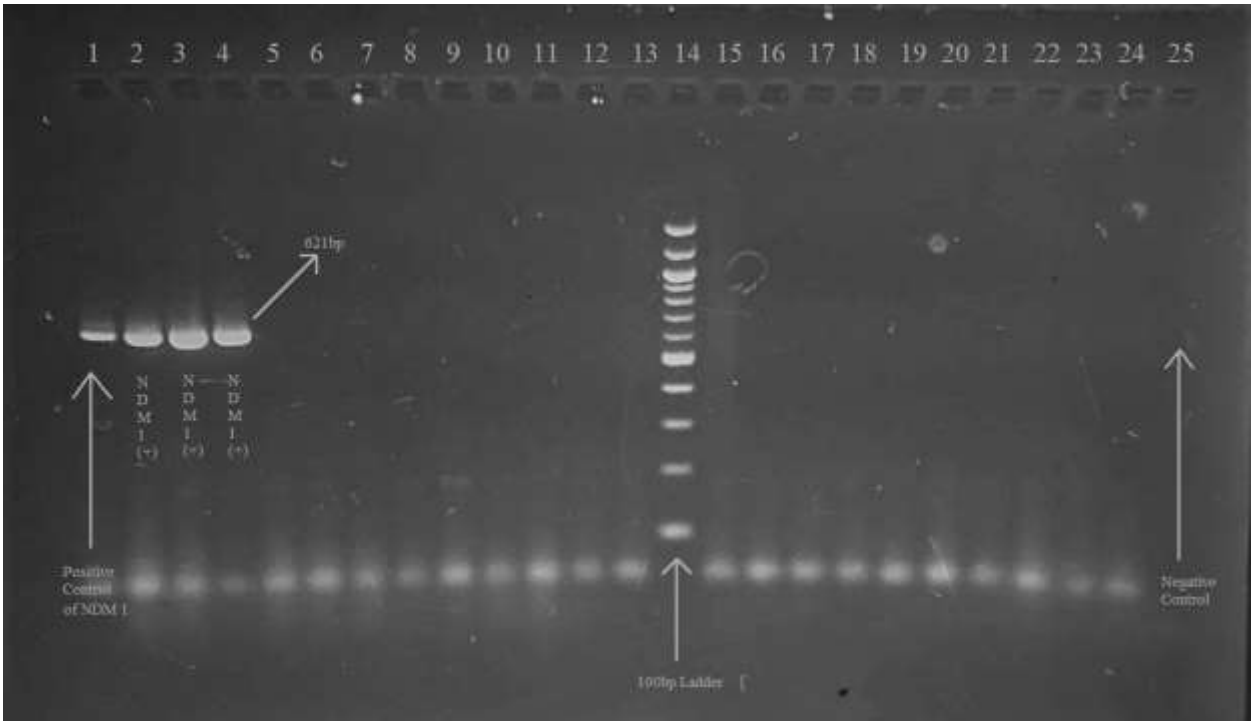


Figure 12: Gel picture of 3 isolates from March Dhaka Shishu Hospital being positive for NDM-1

CHAPTER 5: DISCUSSION

Acinetobacter baumannii is an opportunistic pathogen which is found widely in hospitals, causing all sorts of nosocomial infections. It seems to have an inclination towards developing antibiotic resistance expidtiously, a reason for which could be the fact that it has been exposed to antibiotics for a long time span causing it to evolve for survival. (Gupta et. al, 2015) Thus, it makes *A. baumannii* one of the most prevalent human infection causing bacteria in the hospital setting, as well as a reservoir of MDR genes. Moreover, due to unsystematic prescription of antibiotics in the hospital, this organism is furthur developing resistance against the common antibiotics. (Basatian-Tashkan et al., 2020) The treatment of this bacteria, especially the strains that are MDR and have beta-lactamase activity is crucial. While carbapenems are the choice of drug for treating MDR *A. baumannii*, the number of CRAB are rising steadily. (Santajit et al., 2023) It was found from other studies that *A. baumannii* shows its resistant charecteristics to most of the beta lactams, quinolones and aminoglycosides (Basatian-Tashkan et al., 2020). It has versetile resistant mechanisms against penicillins, cephalosporins, carbapenems and tetracycline (Kyriakidis et al., 2021b). Hospitals accumulate such strains of *A. baumannii* via patients and thus hospital sewage has become a hotspot of habitating a large number of MDR *A. baumannii* which can further disseminate into the environment and end up in our supply water. *A. baumannii* has a large prevalence rate and the antibiotic susceptibility pattern differs in different geographical locations which makes the surveillance of *A. baumannii* in different niches and parts of the world a very important task. (Santajit et al., 2023)

In this study, we isolated *Acinetobacter baumannii* from hospital wastewater and adjacent community tap water near the selected hospitals in order to observe the antimicrobial resistance pattern of these isolates against antibiotics of various classes such as broad spectrum β-lactams, fluoroquinolones, aminoglycosides, carbapenems, 3rd and 4th generation cephalosporins, macrolides, monobactams and penicillins. From the phenotypic analysis we further investigated the presence of different antibiotic resistant genes, namely CTX-M, SHV, TEM, OXA-48, NDM-1 and IMP.

Within the study period of March 2023 to June 2023, a total of 37 samples were collected for our research, out of them 9 were from Hospital waste water and 28 were from adjacent community tap water. For collection of sample three hospitals were selected. They are Dhaka Shishu Hospital, National Cancer Research Institute & Hospital and DNCC COVID-19 Dedicated Hospital. For collecting community tap water, different four houses were selected from a particular radius of area of National Cancer Research Institute and Hospital and Dedicated COVID-19 Hospital as well as for Dhaka Shishu Hospital two houses were selected by the same process for collecting community tap water. Our findings revealed 100 isolates out of 145 presumed isolates to be positive for *A. baumannii* by the detection of intrinsic blaOXA-51 gene. As other studies have

proven, blaOXA-51 is an oxacillinase present in all *A. baumannii* isolates which can be taken as an indication of carbapenem resistance- however merely its presence is not enough to solidify whether the strain is carbapenem resistance. Thus it is used as a way to confirm the identity of *A. baumannii*. (Hassan et al., 2021) According to a study, which was conducted for isolating *A. baumannii* from hospital wastewater effluents, they found 53 confirmed *A. baumannii* from three selected hospitals (Mapipa et al., 2022). In our study we found 31 confirmed *A. baumannii* isolates from hospital wastewater. Among them, 15 out of 31 were from Dhaka Shishu Hospital, 14 out of 31 were from National Cancer Research Institute and 2 out of 31 were from Dedicated COVID-19 Hospital. Here is a mentionable thing, although being in the almost same area, National Cancer Research Institute wastewater has more number of positive isolate (14 isolates) than Dedicated COVID-19 Hospital wastewater (2 isolates) which could be due to the number of patients admitted there.

After doing AST, it was seen that all of the 52 representative *A. baumannii* isolates were resistant to 4 or more antibiotics. All isolates were always resistant to cefixime and ceftazidime which are 3rd generation cephalosporins and additionally the isolates were seen to show resistance against at least 4 of the 11 antibiotics tested against, which makes them classifiable as MDR. This is in accord with another research (Basatian-Tashkan et al., 2020b) where Ceftazidime resistance was seen at 98.4% which is close to our findings. Another previously published research (Aliakbarzade et al., 2014) accounted for 100% resistance against cefixime which matches with our findings, however, there was a difference seen in the resistance pattern of gentamycin and amikacin- where they saw resistance to gentamicin 86% and amikacin 81% while ours showed 5.8% in gentamycin and 3.8% to amikacin. This shows that our isolates had low resistance to aminoglycosides. The sample of their study however, was clinical samples while our study was environmental samples. In another study by (Shahcheraghi, 2011) using clinical samples, a similar high percentage of resistance was seen for aztreonam (96%), ceftazidime (86%) and cefexime (100%) while our study showed aztreonam (84.6%), ceftazidime (96.1%) and cefexime (100%). Their isolates also showed high resistance for amikacin (84%) and cefepime (90%) while ours showed low resistance amikacin (3.8%) and cefepime (13.4%). For both clinical studies, high resistance was seen against carbapenems while ours was only 9.6% for imipenem. In a Bangladesh- based study by (Farzana et al., 2022) all their environmental *A. baumannii* isolates were completely resistant to imipenem, meropenem, gentamicin, amikacin, and ciprofloxacin which is a reverse situation in our case where imipenem, gentamicin, amikacin were seen to have high sensitivity instead of resistance. However all their environmental strains were MDR which aligns with our findings. A similar research (Islam et al., 2017) showed their wastewater isolates against gentamicin (94%) and amikacin (81%) conferring high aminoglycoside resistance. In Ferreira et al. (2011b)'s study conducted in Brazil, *A. baumannii* extracted from wastewater samples were mostly susceptible to carbapenems- : imipenem (95.14%) and meropenem (85.39%) while 84.6% of our isolates of our study were susceptible to imipenem. The uninhibited use of broad-spectrum cephalosporins in hospital settings as they are less toxic in comparison to other antibiotics and they are always readily

accessible in pharmacies has led to the emergence of *A. baumannii* that are resistant to these antibiotics, as also observed in our conducted study.

The results of the genotypic detection resistant genes found in our isolates showed little relationship with the phenotypic assessment after AST. As seen in our study, Almost all isolates were resistant to cefixime (100%) and ceftazidime (96%) which are 3rd generation cephalosporins and aztreonam (84.6%). This indicates that there should be a presence of an ESBL encoding gene among the 3 main genes we tested for- CTX-M, SHV and TEM. However, the presence of these genes were not found in any of the isolates after PCR detection. The ESBLs are known to be able to hydrolyze 3rd and 4th generation cephalosporins and aztreonam. However, all phenotypically detected ESBL producing isolates in our study did not show the presence of ESBL encoding genes. Thus, other mechanisms such as efflux pumps, target site alteration, membrane permeability and hyper-production of cephalosporinase possibly are involved. Also, so far above 350 natural ESBL variants are present to our knowledge and these have been divided into nine categories based on structure evolution and their amino acid sequence comparisons such as TEM, SHV, CTX-M, PER, VEB, GES, BES, TLA, and OXA. (Bajpai et al., 2017) Among these we only tested 3, so it could be that these isolates had any of the other genes that were not tested. Even among OXA-types, there is OXA-23, 24, 40, 48, 58, 72 and only OXA-48 was tested so it remains to be seen whether other genes were present.

In our study we selected 52 nonduplicate isolates to check for AMR genes. We found 5 NDM-1 positive isolates. Among them 4 were from Dhaka Shishu Hospital waste water and 1 was from National Cancer Research Institute community tap water. No other gene tested for was found in our isolates. There a study was conducted in Beijing, China where they tried to isolate NDM-1 producing *Acinetobacter baumanni*i from the sewage of 4 hospitals and found 10 NDM-1 isolates (Zhang et al., 2013). Similarly, a study conducted in Bangladesh where samples were taken from wastewater adjacent to hospitals found the presence of NDM-1 positive *A. baumannii* isolates [n=8]. (Islam et al., 2017b) The NDM-1 gene has been characterized to be a big problem for public health ever since it was identified in 2009. Bacteria carrying this gene are seen to be resistant to nearly all antibiotics. Thus it is crucial to be able to detect and monitor these microorganisms crucially. (Hubeny et al., 2022d)

Middle Eastern countries have conducted studies which show that there is a spike *A. baumannii* strains that are resistant to cephalosporin and it is mainly due ESBL production through acquiring blaCTX-M, blaSHV, blaGES, blaOXA, and blaNDM β-lactamase genes. For example, a study from Iraq which was on detection of *A. baumannii* from the hospital environment, out of 21 isolates, most of the isolates were positive for CTX-M gene and TEM gene. They found SHV positive in 3 isolates and IMP positive in 1 isolate. But they did not find any isolates that are positive for OXA (Al-Kadmy et al., 2018). But from our study we did not find any positive for CTX-M, SHV, TEM, OXA-48 and IMP. A large number of studies have shown MDR *A.*

baumannii having the OXA-23 gene. (Kovačić et al., 2017), (Hrenović et al., 2016), (Farzana et al., 2022b), (Ferreira et al., 2011c), (Santajit et al., 2023b), (Al-Haddad et al., 2018), (Safari et al., 2015b). OXA-23 is widespread in patients and hospital environments of previously examined hospitals of Bangladesh. (Farzana et al., 2022b) CRAB isolates were also seen to carry OXA-40 (Kovačić et al., 2017), (Hrenović et al., 2016). Though most of these studies were conducted with clinical samples rather than environmental samples. This however highlights how widespread these genes are among the hospital so it can be easily available in hospital wastewater and dessiminate to the community.

5.1 Limitations of our study

Our study was limited to 3 hospitals in Dhaka-north so it could not reveal the whole picture of the scenario of wastewater microbes. This investigation could also be improved greatly from using more relevant antibiotics to treat the isolates; however, it was constrained due to laboratory unavailability. Besides due to problems with antibiotic resistant gene primers in blaKPC, VIM and well as unavailability of primers for OXA-23, 40, 58 we could not report a full picture of molecular characterization of AMR bacteria and the antibiotic resistance genes they potentially possess. By utilising new forms of molecular assays such whole genome sequencing, the isolates of *A. baumannii* recovered from wastewater should be compared with hospital and the community supply waters, thus further study is needed for the genotypic characterization and to justify whether horizontal gene transfer, conjugation or transduction is actually happening or not between isolates of hospital wastewater and adjacent community tap water.

To start any response plan for the environmental control of *A. baumannii* pollution, the source and future spread must be tracked. By utilising new forms of molecular assays such whole genome sequencing, the isolates of *A. baumannii* recovered from wastewater should be compared with hospital and the community supply water. Moreover veterinary/livestock, and community-acquired strains, where there may be discernible variations between these strains can be analysed. To learn more about how human waste affects the transmission of MDR *A. baumannii* in the environment, larger screening and epidemiological investigations should be conducted. Understanding the epidemiology of this human pathogen is crucial in the current period, when new antibiotics for MDR Gram-negative germs are hard to come by. The study's findings are a positive development.

CHAPTER 6: CONCLUSION

The present study highlights that there is an availability of NDM-1 producing *A. baumannii* in the hospital wastewater of one of Dhaka's most busy hospitals, as well as found from community tap water which is quite alarming. Even though the antibiotic resistance profile shows a low % resistance to imipenem which is a carbapenem used to treat MDR *A. baumannii,* an extremely high % i.e almost all isolates retrieved were resistant to cefixime and ceftazidime which is an alarming indication to beta-lactam resistance which is one of the most widely used antibiotics for treating *A. baumannii* infection. Hospital waste that hasn't been treated is a significant source of these microorganisms. Our findings have influence on public health that are certainly important. Although the majority of the samples used in this study were from Dhaka, Bangladesh, a comparable situation might exist in any setting in a developing country where there is a high prevalence of MDR organisms among patients as a result of unchecked antibiotic use, inadequate sanitation and hygiene, and subpar waste management policies. This study also emphasises the critical need for installing effective wastewater treatment facilities in healthcare settings as part of biosecurity programmes, as well as the necessity of continuously monitoring hospital wastewater for antibiotic-resistant bacteria. The presence of multiresistant NDM-1 producing strains of *A. baumannii* in hospital wastewater and community tawater from these hospitals indicates that the pathogenic strains which circulate in the hospital environment can reach the wastewater and can, therefore, enter local water supply which necessitates and highlights the need for more work to be done to understand the transmission of MDR bacteria.

CHAPTER 7: REFERENCES:

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